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Development of Human Cytochrome P450 Competent Genotoxicity Tester bacterial Systems for High Throughput Screening

Bernardo J.E. de Brito Palma

2017

Development of human Cytochrome P450 competent genotoxicity tester bacterial systems for high throughput screening

Functional characterization of human Cytochrome P450 1A2 polymorphic variants

Bernardo J. E. de Brito Palma

INVITATION

Thesis defense

Bernardo Brito Palma

Development of human Cytochrome P450 competent genotoxicity tester bacterial systems for high throughput screening

Functional characterization of human Cytochrome P450 1A2 polymorphic variants

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Monday,
16th October 2017
at 13.45 hr

Aula
Vrije Universiteit
De Boelelaan 1105
Amsterdam

Reception will follow afterwards

bernardo.palma@nms.unl.pt

Paranimfs
Jelle Reinen
Patrícia Pereira

Development of human Cytochrome P450 competent genotoxicity tester bacterial systems for high throughput screening

Functional characterization of human Cytochrome P450 1A2 polymorphic variants

Bernardo J.E. de Brito Palma

The research described in this thesis was primarily carried out at the Department of Genetics, Faculty of Medical Sciences (currently, Department of Genetics, Oncology and Human Toxicology, NOVA Medical School), Universidade NOVA de Lisboa, Campo dos Mártires da Pátria, 130, 18 1169-056 Lisboa, Portugal. Part of it was carried out in the Division of Molecular Toxicology, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, Vrije Universiteit Amsterdam, currently AIMMS, De Boelelaan 1108, 1081 HZ Amsterdam, The Netherlands.

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VRIJE UNIVERSITEIT

Development of human Cytochrome P450 competent genotoxicity tester bacterial systems for high throughput screening

Functional characterization of human Cytochrome P450 1A2 polymorphic variants

ACADEMISCH PROEFSCHRIFT

ter verkijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Bètawetenschappen
op maandag 16 oktober 2017 om 13.45 uur
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De Boelelaan 1105

door

Bernardo José Espadinha de Brito Palma

geboren te Lissabon, Portugal

promotoren:	prof.dr. N.P.E. Vermeulen
	prof.dr. J. Rueff
copromotor:	dr. M. Kranendonk

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dr. R. van der Oost

aos meus Pais, e minha mulher Patrícia

para os meus filhos, Frederico e Margarida

"This is the way of the world, but is not the only way."

Drizzt Do´Urden

(R.A. Salvatore)

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PART I

INTRODUCTION

CHAPTER 1

General introduction, scope and objectives

1.1. XENOBIOTIC EXPOSURE

Humans are exposed to a myriad of xenobiotics, such as pharmaceuticals, cosmetics, dietary, environmental or occupational chemicals. Moreover, an increasing number of chemicals are newly generated by isolation, purification or combinatorial chemistry techniques, causing a serious challenge when it comes to toxicological evaluation. This challenges became even more evident as result of the implementation of the EU chemical policy designated REACH in 2006 [1]. Toxicological testing protocols usually comprise both *in vivo* and *in vitro* assays, using laboratory animals, and organ-, tissue- and cell-systems, respectively, or *in silico* predictions. The *in vivo* (animal) studies have been increasingly been recognized to suffer from disadvantages such as high costs, time consuming, the need of many animals as well as the ethical aspect of the use of laboratory animals [2]. *In vitro* organ-, tissue- and cell-models are of particular interest due to their relative simplicity in manipulation, the collection of data in short term, being in line with the societal concern of laboratory animal use. In 2007, the US National Research Council (NRC) as proposed a new long-range vision for toxicity testing and risk assessment [3]. In this document, is proposed to increase the resource to *in vitro* toxicity pathway assays in detrimental of the usual qualitative *in vivo* systems, using high-throughput screening with mechanistic quantitative parameters. As so, significant perturbations in these toxicity pathways would be the focus for risk assessment. It is also suggested that *in silico* methods should be implemented to determine the dose-response models of perturbations of pathway function [4].

In xenobiotic risk assessment or drug development, the preliminary analysis of compounds for carcinogenicity often relies on genotoxicity or mutagenicity testing. The currently used mutagenicity (*in vitro*) assays are considered to suffer from two main drawbacks, namely relatively tedious procedures and long duration and the lack of

(human) biotransformation, leading to limitations in efficiency and reliability. The currently standard *in vitro* approaches may fall short due to the inadequate representation of chemically reactive metabolites (CRMs) formed by (human) biotransformation [5]. Biotransformation and its inter-individual variability, play a major role in chemical genotoxicity, carcinogenicity and susceptibilities.

1.2. GENERAL ASPECTS OF BIOTRANSFORMATION

The elimination of xenobiotics habitually depends on their conversion to hydrophilic molecules by a process known as biotransformation (or metabolism), which is catalyzed by enzymes mainly in the liver but also in other tissues [6]. Absorption through the skin, lungs or gastrointestinal track of many xenobiotics is facilitated by lipophilicity, which is also an obstacle for their elimination, since they can be readily reabsorbed. In general, xenobiotic metabolizing enzymes catalyze the formation of products (metabolites) that are more water-soluble (hydrophilic) than the parent compound, thereby favoring excretion in urine, bile or feces [7]. Metabolism, in most of the cases, results in suppression of biological activity, as the metabolites, in contrast to the parent compounds, are often unable to reach their site of action and/or fail to interact with the correct targets [8]. Thus, biotransformation plays a major role in protecting the organism from possible toxic effects of xenobiotics.

Recently, the concept ‘metabolic activation or bioactivation’ of drugs and other chemicals, is becoming better understood. Bioactivation of parent compounds into products with more biological activity, possibly being ‘pro-drug activation’ [9], the metabolite having the same or different pharmacological effects as the parent drug [10]. However, the metabolic activation or bioactivation of xenobiotics may also result in the formation of CRMs, which are known to play a role in multiple toxicities, and in

genotoxicity in particular [11, 12]. For about 75-80% of current drugs and other xenobiotics, metabolism is the major clearance pathway and is a key determinant in the performance and safety of drugs and other xenobiotics [13, 14]. The liver is the main site of xenobiotic biotransformation, although it also occurs in other organs and tissues. For the majority of the 200 drugs often prescribed in the USA, the routes of elimination were found to be hepatic [15].

Xenobiotic biotransformation processes are usually separated into three categories, namely phase I, phase II and phase III. Phase I reactions involve the introduction (or modification) of a functional group by hydrolysis, reduction and oxidation, normally resulting in a small increase of hydrophilicity of the compounds. Phase II reactions result in the conjugation with an endogenous polar cofactor, normally resulting in a greater increase in hydrophilicity. These conjugation reactions, include glucuronidation, sulfation, acetylation, methylation, glutathione- and amino acid-conjugation [6]. Finally, Phase III reactions/processes involve transporters, such as, P-glycoprotein (P-gp), organic anion transporting polypeptides (OATPs) and multidrug resistance proteins (MRPs), which export the products formed by phase I and II reactions [16].

In phase I metabolism, cytochrome P450 (CYP) family is the major enzymatic system involved, being accountable for about three quarters of the total metabolism of marketed drugs (see Figure 1) [17]. The CYP enzymatic system is further described below, since will be object of study in the work presented in this thesis. The remaining phase I metabolism is carried out by a variety of other contributing enzymes, including Flavin-containing monooxygenases (FMOs), NADPH:quinone oxidoreductases (NQOs), and several others such as dehydrogenases, esterases and oxidases [17, 18].

In phase II metabolism the main contributors are UDP-glucuronosyl transferases (UGTs), complemented by sulfotransferases (SULTs), N-acetyltransferases (NATs) and glutathione S-transferases (GSTs) (see Figure 1) [19]. Phase II enzymes will not be further discussed since they are not relevant in the context of this thesis.

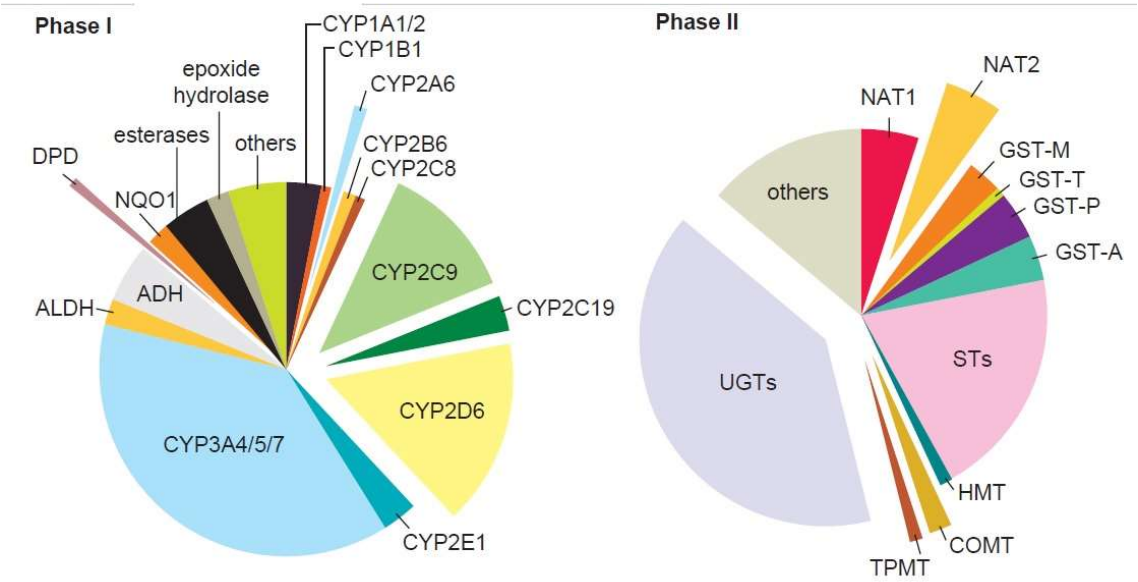

Figure 1. Overview of the main phase I and phase II enzymes involved in xenobiotic metabolism in humans. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH:quinone oxidoreductase; COMT, catechol O-methyltransferase; GST, glutathione-S-transferase; HMT, histamine methyltransferase; NAT, *N*-acetyltransferase; ST, sulfotransferase; TPMT, thiopurine methyltransferase; UGT, uridine 5′-triphosphate glucuronosyltransferase. Adapted from [18].

1.2.1 Polymorphisms and interindividual differences

Inter-individual variability in xenobiotic and drug metabolism is extensive. Plasma levels of a drug can vary more than 1000-fold between individuals upon the same drug dosage [20]. Moreover, it has been described that the proportion of patients which respond positively to the first drug prescribed in the treatment of a wide range of diseases is typically just 50–75% [21]. These variations may be due to differences in the expression

of genes responsible for drug metabolism, transport, and response and may be caused by genetic, epigenetic, physiological, patho-physiological, or environmental factors [22]. In fact, it is considered that genetic factors are responsible for 20-40% of the differences between individuals in drug response as well as susceptibility to the toxicity of drugs and xenobiotics [23]. Furthermore, it has been estimated that attrition during drug development for around 12% of the drug candidates is due to genetic safety concerns [24]. Therefore, inter-individual variability in drug response and toxicity may lead to patient harm and ineffective use of limited healthcare resources [25].

Genetic polymorphism include mutations both in non-coding regions (promoter, intron) and protein-coding sequences. Mutations in non-coding regions may lead to altered expression levels, while mutations in protein-coding sequences may change the structure and catalytic properties of the enzyme [26]. Fortunately, the number of genetic biomarkers for the prediction of drug choice and correct dosage are increasing, allowing the personalization of treatment. Some recent genome-wide association studies showed that genetic polymorphism among the drug-metabolizing enzymes can disturb endogenous functions such as blood pressure, suicide risk, and bilirubin levels, as well as exogenous factors like coffee intake, cigarette consumption, drug efficacy and toxicity susceptibility [27]. Such polymorphisms and others, offer a resource of pharmacogenomic biomarkers that can help to optimize drug treatment, particularly in the areas of oncology, cardiovascular disease, infection, and psychiatry [22, 28].

CYP polymorphisms are strongly implicated in human susceptibility to cancers (see Table I) [29-32]. Moreover, CYP polymorphisms have been indicated as a major reason why substantial numbers of patients do not respond, respond partially, or suffer from adverse drug reactions (ADRs) [33, 34]. Therefore, it is fundamental to understand the consequences of CYP polymorphisms, to improve our comprehension of inter-

individual differences in the outcome of drug treatments, as well as of chemical exposure in general [35].

Table I. Examples of CYP polymorphisms related with susceptibility to cancer. Adapted from [29].

CYP allele designation[a]	Key mutation(s)[b] rs number	Location, protein effect	Allele frequencies[c]	Functional effect	Clinical correlations
CYP1A1*2C	2454A>G (rs1048943)	I462V	gMAF 0.120 0.0–0.04 Af, AA 0.20–0.26 As 0.03–0.07 Ca 0.18–0.43 Hs 0.17 Pc	↑ Activity (17β-estradiol and estrone)	↑ Lung cancer risk in Chinese; ↑ breast cancer risk in Caucasians; ↑ prostate cancer risk
CYP1A2*1C	−3860G>A (rs2069514)	Promoter	gMAF 0.188 0.26–0.40 AA, Af 0.21–0.27 As, Pc 0.01–0.08 Ca 0.20–0.30 Hs	↓ Inducibility (smokers)	May influence susceptibility to certain cancers
CYP1A2*1F	−163C>A (rs762551)	Intron 1	gMAF 0.35 (A>C) 0.5–0.8 all ethnicities	↑ Inducibility (smokers, omeprazole)	↑ Susceptibility to cancer in Caucasians; ↑ oral clearance olanzapine; possible modifier for risk of coronary heart disease
CYP1B1*6	142C>G (rs10012); 355G>T (rs1056827); 4326C>G (rs1056836)	R48G A119S L432V	gMAF 0.32–39 0.5–0.85 AA, Af 0.09–0.13 As 0.23–0.40 Ca	↑ Km, ↓ Vmax (17β-estradiol, recombinant)	↑ Prostate cancer risk for L432V in Asians
CYP2A6*2	1799T>A (rs1801272)	L160H	gMAF 0.013 0.00–0.01 AA, Af 0.00–0.025 As 0.04–0.10 Ca	No activity	↓ Nicotine metabolism & influence on cigarette consumption, nicotine dependence, smoking cessation response; ↑ lung cancer risk in Caucasians; ↓ oral clearance of tegafur

CYP3A4*1B	−392A>G (rs2740574)	Promoter	gMAF 0.20 0.50–0.82 AA, Af 0.00 As 0.03–0.05 Ca, Hs, SA	Probably no effect on transcription	↑ Prostate cancer disease progression

[a] According to the CYPallele nomenclature homepage (http://www.cypalleles.ki.se).

[b] Genomic positions are given with corresponding rs numbers in parentheses.

[c] gMAF, global allele frequency of the minor allele as reported in the 1000Genome phase 1 genotype data (released May 2011). Selected frequencies of individual ethnicities (AA, African American; Af African; As Asian; Ar, Arab; Ca Caucasian; Hs, Hispanic; In, Indian; Pc, Pacific; SA, South American) were compiled from dbSNP (build 137) at http://www.ncbi.nlm.nih.gov/projects/SNP/; from the Allele Frequency Database ALFRED at http://alfred.med.yale.edu/alfred/index.asp

1.3. CYTOCHROME P450

CYP enzymes are the major catalysts involved in the biotransformation of xenobiotics and are responsible for the metabolism of a wide variety of clinically, physiologically and toxicologically important compounds [36]. The name of CYP is derived from the characteristic presence of an absorption maximum at 450 nm in the differential spectra of reduced CO-bond complex [37]. The CYP superfamily consists of 57 genes (and 58 pseudo-genes) and is divided into 18 families and 44 sub-families in humans, involved in the synthesis of endogenous compounds and the metabolism of drugs and other xenobiotics [38-40]. The CYP families 1, 2 and 3 are accountable for 70-80 % of all phase I metabolism of clinically used drugs and are involved in the biotransformation of a large number of drugs and other xenobiotics [29, 33]. In fact, five specific members namely, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5, are responsible for about 95% of all CYP-catalyzed drug metabolism [17]. The majority of CYP-mediated metabolism is carried out by polymorphic enzymes [41]. All genes that

encode CYPs from families 1-3 are highly polymorphic (see *http://www.cypalleles.ki.se/)* [29, 42].

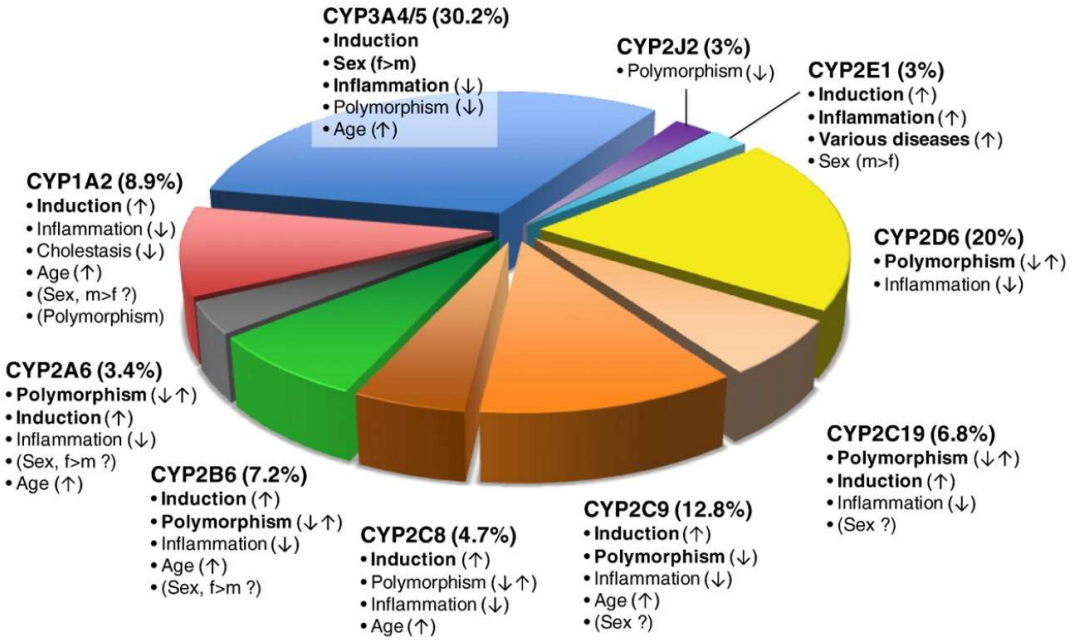

Figure 2. CYP isoforms contribution to drug metabolism and factors influencing variability. A total of 248 known CYP-dependent drug metabolism pathways were analyzed. Variability factors are indicated in bold with possible directions of influence indicated (↑, increased activity; ↓, decreased activity; ↑↓, increased and decreased activity). Factors with controversial significance are shown between parentheses. Adapted from [29].

The primary reaction catalyzed by CYPs is mono-oxygenation, incorporating a single oxygen atom, from molecular oxygen, into a substrate (designated RH, Figure 3), while the other oxygen atom is reduced to water [6]. When CYPs act as a mono-oxygenase, the products formed are not limited to alcohols and phenols, as a result of re-arrangement reactions [43]. Thus, as a result of the main mono-oxygenase activity of CYP, in the presence of oxygen, drugs and other chemicals undergo different types of reactions, such as hydroxylations, epoxidations, heteroatom dealkylations, deaminations, dehydrogenations, oxygenations, esters cleavages, and others. On the contrary, under anaerobic conditions and in presence of reducible substrates, CYPs may act as a

reductase, e.g. resulting in azo-reduction, nitro-reduction and reductive dehalogenation [6, 44].

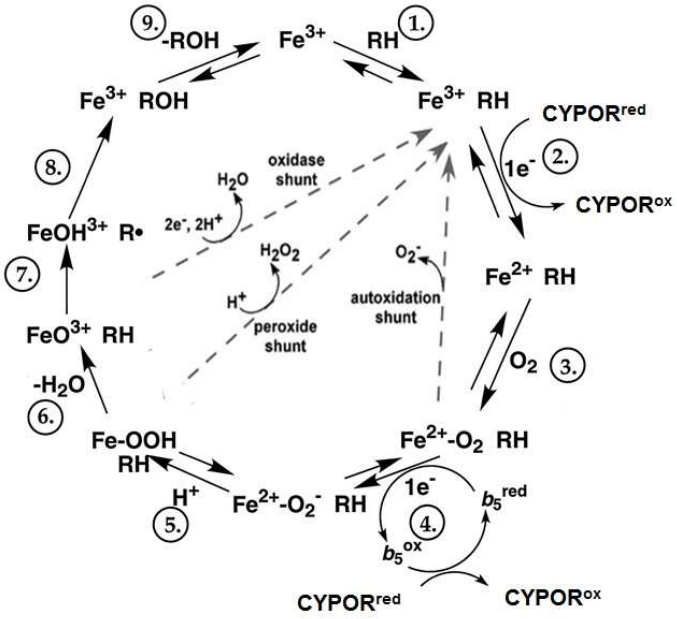

Figure 3. Cytochrome P450 (CYP) general catalytic cycle. Individual steps in the cycle include: (1) substrate binding; (2) first one-electron reduction by CYPOR; (3) oxygen binding; (4) second one-electron reduction by CYPOR or b_5; (5) protonation of the distal oxygen coordinating to iron; (6) water molecule abstraction, with formation of the reactive heme iron–oxygen species; (7) radical intermediate formation; (8) oxygen rebound with the radical intermediate; (9) product release. The cycle can be short-circuited in three different places without product formation, namely, autoxidation shunt, peroxide shunt and oxidase shunt. Adapted from [45] and [46]

During the catalytic cycle, auto-oxidation may occur, whereby the cycle is shunted and molecular oxygen is reduced (released as a free radical) without product formation, known as uncoupling (autoxidation shunt) (see Figure 3). Furthermore, this uncoupling may occur in the presence of artificial oxygen delivery agents, such as peroxides (peroxide shunt) (see Figure 3) [36], or when the reactive heme iron–oxygen species intermediate is oxidized to water instead of oxygenation of the substrate (oxidase shunt) (see Figure 3) [46]. The accumulation of reactive oxygen species (ROS) can contribute

to free radical propagation [47] and ultimately leading to DNA adduct formation and to the development of cancer [48].

1.3.1 CYP1A2 and bioactivation

The human CYP1A subfamily consists of CYP1A1 and CYP1A2. CYP1A1 is mainly expressed in extra-hepatic tissues while CYP1A2 is almost exclusively expressed in the liver, representing about 13-15% of its total CYP content [49]. These enzymes are important catalysts for the bioactivation of several precarcinogens, such as polycyclic aromatic hydrocarbonates (PAHs) as well as aromatic and heterocyclic amines (see Figures 4 and 5 for examples) [50]. Actually, the CYP1 family is strongly implicated in the development of cancer [51-54].

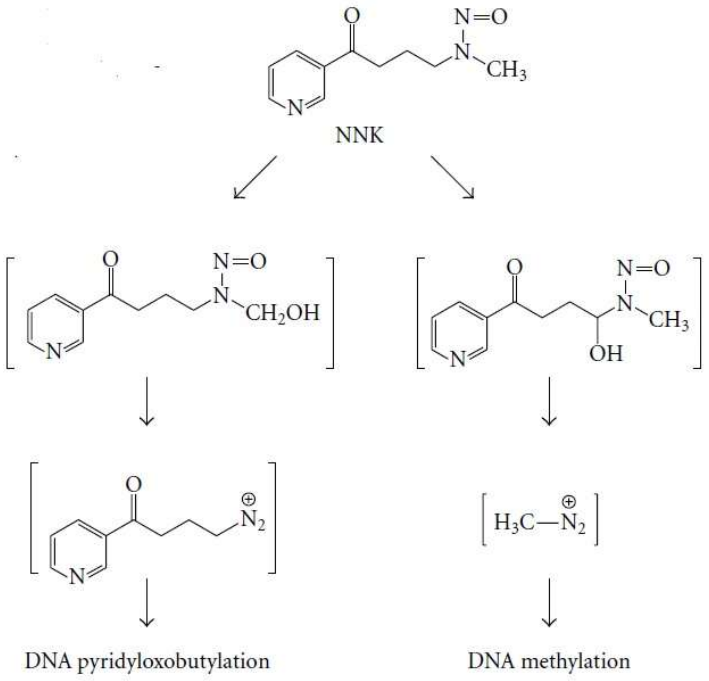

Figure 4. CYP1A2 mediated bioactivation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is metabolized to either a methylating or a pyridyloxobutylating agent. Adapted from [55].

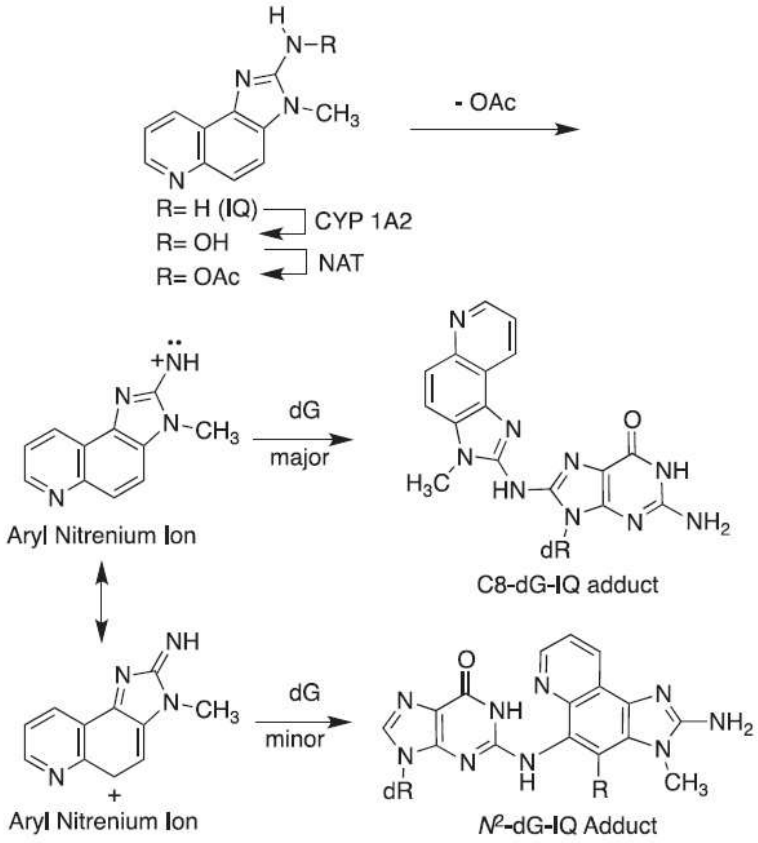

Figure 5. CYP1A2 mediated bioactivation of 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ). CYP1A2 mediates the N-hydroxylation of IQ, which is followed by N-acetylation of the hydroxylamine and deacetylation forms an electrophilic aryl nitrenium ion. The aryl nitrenium ion alkylates guanine in DNA. This occurs either via the IQ amine nitrogen to form the C8-dG-IQ adduct, or alternatively, alkylates DNA via the C5 position of the IQ ring to form the N2-dG-IQ adduct. Adapted from [56].

The CYP1A2 crystal structure [57] (PDB file ID 2HI4) demonstrated a relatively planar and small binding cavity, with an estimated volume of about 375 Å [58], fitting closely with planar compounds, such as the typical CYP1A2 substrates, theophylline and caffeine, and the potent inhibitor α-naphthoflavone (see Figure 6) [59, 60]. CYP1A2 mediates the metabolism of about 15% of clinical drugs [61], such as clozapine [62, 63], lidocaine [64], phenacetin [65, 66], propranolol [67] and tacrine [68].

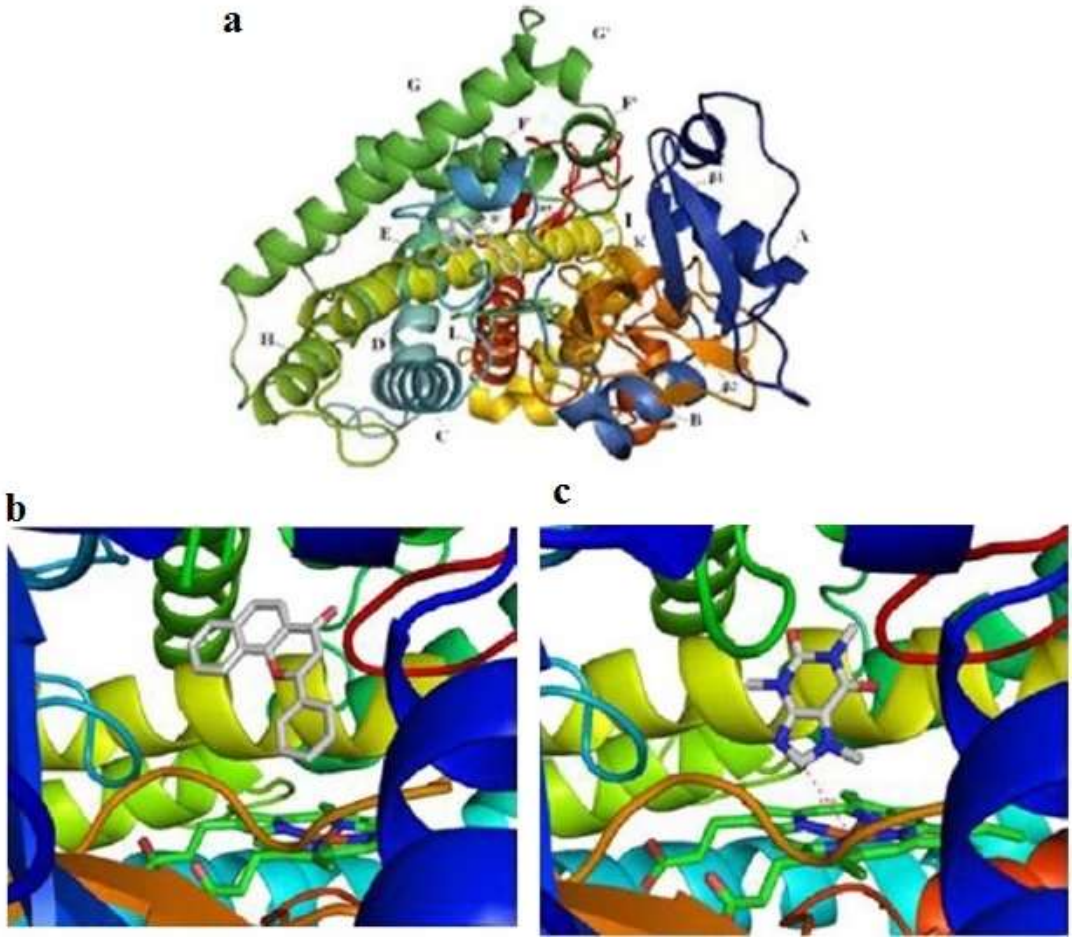

Figure 6. Overview of the structures of human CYP1A2 with co-crystallized ligand α-naphthoflavone (α-NF) (PDB file ID 2HI4) (**a**). Image zoomed in on the active center in complex with α-NF (**b**) or with caffeine (**c**). The metabolic site of caffeine is 4,34 Å apart from the heme. Adapted from [60].

CYP1A2 is a polymorphic enzyme and to date, 41 CYP1A2 haplotypes have been designated on the Human Cytochrome P450 Allele Nomenclature Committee home page (*http://www.cypalleles.ki.se/cyp1a2.htm*). The inter-individual differences in CYP1A2 activity are well known: up to 60-fold variations have been reported [49, 69]. These differences are most likely to influence drug metabolism, and thus have clinical relevance in drug efficacy and safety; and alter susceptibility in exposure to (pre)carcinogens. Determinants such as diet [70], smoking [71] and intake of oral contraceptives [72] are well established modifiers of CYP1A2 activity. In turn, genetic factors were suggested to

represent about 35-75% of the inter-individual variability for CYP1A2 activity [73, 74]. CYP1A2 polymorphisms could also be related with observed toxicity of leflunomide in rheumatoid arthritis patients [75] and seem to play a role in efficacy of the treatment of patients with antipsychotics, such as clozapine and olanzepine [76]. Several recent studies indicated implications of CYP1A2 polymorphisms in cancer susceptibility, namely for pancreatic cancer [77, 78], and bladder cancer [79].

1.3.2 Cytochrome b_5 as CYP redox partner.

To carry out catalysis microsomal CYP metabolism requires a coupled supply of 2 electrons, which are normally provided by the auxiliary protein NADPH cytochrome P450 oxidoreductase (CYPOR) [80]. As CYP, CYPOR is a membrane-bound protein which contains two flavin moieties namely, FMN and FAD, essential for sequential one electron transfer to CYP, prevenient from NADPH [46]. For at least 4 decades, another heme protein, namely cytochrome *b_5* (b_5), has been recognized to alter the rate of catalysis by selected CYPs [74, 81-83]. The discussion concerning b_5 involvement in CYP reactions has been controversial, since it was first suggested by Hildebrandt and Estabrook in 1971 [84].

The cytochrome protein *b_5* is a ubiquitous eukaryotic small protein, of approximately 17 kDa, which is present throughout the phyla and appears to be an essential component of a number of endoplasmic reticulum-linked redox enzyme systems [85, 86]. Physiologically, b_5 can be reduced by both cytochrome *b_5* reductase (NADH dependent), and by CYPOR [87]. It has been reported, that b_5 is able to enhance, inhibit, or not alter the catalysis of CYPs depending on the substrate, the specific CYP isoform and the experimental conditions [88-90]. This non-consistent influence of b_5 is now better understood after the demonstration that b_5 is able to catalyze product formation faster

than CYPOR, under single turnover conditions, which gives less time for side product formation, and also that b_5 and CYPOR compete for a binding site on the proximal surface of CYP [91]. Several studies demonstrated that b_5 could modulate specific activities of several CYPs. Effects of b_5 are reported in the catalytic properties of human CYP1A1 [92-94], CYP1A2 [92, 95], CYP3A5 [96], CYP3A4 [96-99], CYP2C9 [97, 99], CYP2B4 [82], CYP2A6 [95, 97] and CYP2E1 [95, 97, 100, 101].

Although substantial studies have been reported on the function and effect of b_5 on CYP-mediated catalysis, during the last decades its mode of action is still not fully understood. Furthermore, analyzing the effects of apo-b_5 on CYPs catalytic properties, it seems plausible that b_5 also plays a conformational role in the human CYP enzymatic system [97, 102]. It is now generally accepted that b_5 acts as an allosteric factor which facilitates the interaction between CYPOR and CYP, and may act as an alternative donor for the second, but not the first, electron in the CYP cycle (see Figure 3) [103]. However, recently it was demonstrated, using extreme *in vitro* conditions, that b_5 is able to supply both electrons to CYP, prevenient from NADH [104].

1.4. CHEMICALLY REACTIVE METABOLITES: BIOACTIVATION AND BIOINACTIVATION

Xenobiotics are body-foreign compounds (such as drugs, pollutants, cosmetics, food, etc.), which normally are not produced or expected to be present in the organism. Biotransformation of xenobiotics normally constitutes detoxification (bio-inactivation) and may involve a large number of proteins. In some cases, bio-activation of xenobiotics may occur, resulting in the formation of CRMs, that are of major importance in drug development and toxicology. CRMs can cause several unwanted effects, such as covalent binding to proteins and DNA, inducing multiple organ toxicities (the liver being the organ

most frequently affected) (see Figure 7). These alterations may induce mutagenesis which can result in tumour formation, immune mediated hypersensitivity reactions and CYP inhibition leading to drug-drug interactions [105].

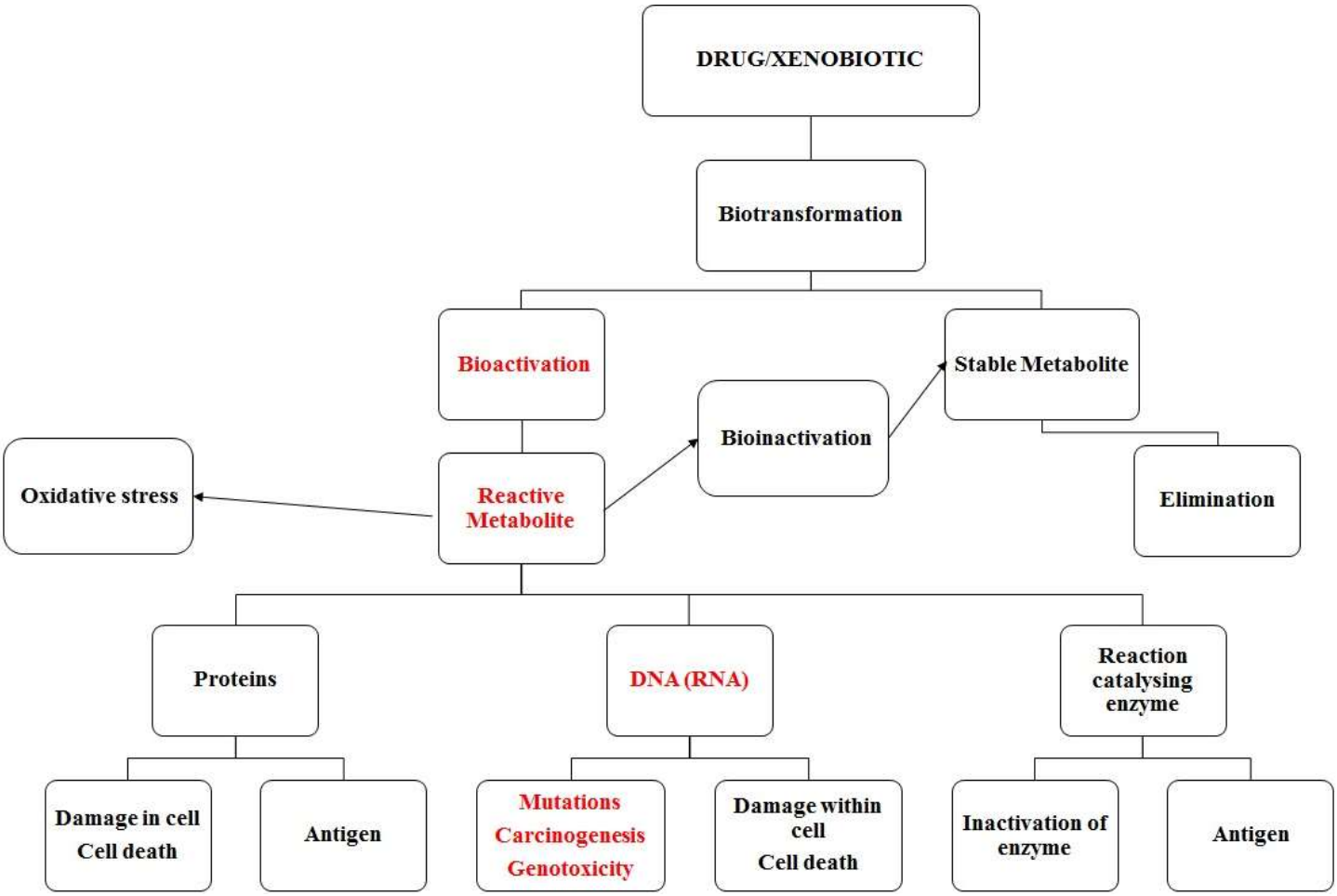

Figure 7. Schematic view of possible causal relations between CRMs and toxicity outcomes. Genotoxicity, important to this thesis, is highlighted in red. Adapted from [11] and [106].

The association between CRM formation and drug toxicity is complex [107]. Most toxicities are dose dependent, and can readily be replicated in various preclinical species including in human systems and are referred to as Type A toxicities. A classic example is hepatotoxicity caused by the formation of NAPQI, a quinone-imine derived from acetaminophen (paracetamol) metabolism (see Figure 8a) [108]. In other cases, toxicity

in man is an unpredictable and relatively rare event. In this so-called idiosyncratic toxicity (iADR), often referred to as Type B toxicity, dose dependence in a population is not clear and cannot be easily replicated in preclinical species. However, while they may not seem to be dose dependent in a broad human population, it does not mean there is no dose dependency in the adverse outcome [12]. An example of Type B idiosyncratic toxicity is agranulocytosis caused by the covalent binding to proteins of the diazatropylium ion, a CRM formed upon clozapine bioactivation (see Figure 8b) [109].

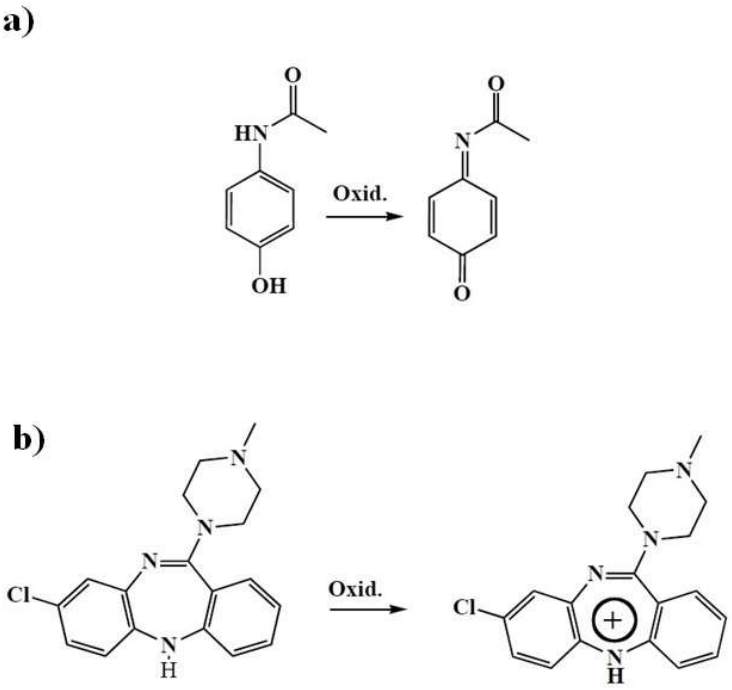

Figure 8. a) Acetaminophen (paracetamol) is bioactivated by CYP to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). **b)** Clozapine is bioactivated by CYP to form the reactive diazatropylium ion. Adapted from [12].

Testing for the propensity of compounds to form CRMs has increasingly become integral part of the absorption, distribution, metabolism, excretion, and toxicity (ADMET) guided lead-optimization in drug discovery and development. To avoid costly late stage failures predictive toxicology assays and models are being utilized as part of a

'fail early' or avoidance strategy [110]. Although *in silico* tools for identifying structural alerts is currently a standard practice at the lead optimization/candidate selection stage of drug development, it is imperative to demonstrate experimentally whether predicted structural alerts are actually are prone for CRMs formation [111]. *In vitro* data is additionally used in early metabolite profiling of CRM-positive candidates which can possibly assist in further prioritization and ranking of compounds during lead optimization/candidate selection of drug development [112].

Traditionally the propensity of drug candidates and their bioactivated CRMs to irreversibly bind to cellular targets (such as: DNA, proteins, lipids, etc. See Figure 7), is performed by covalent binding assays (CB) [113]. LC-MS analysis of trapped CRMs using low molecular weight trapping agents, such as GSH or GSH-analogues, is a commonly used method [114, 115]. Though, it has been observed in *in vitro* studies [114] that even non-toxic drugs can occasionally produce significant amounts of GSH-conjugates, indicating that GSH-conjugates formation *per se* is not predictive for toxicity [116]. Moreover, GSTs play an important role in bio-inactivation of several drugs, such as clozapine, since the spontaneous conjugation of CRMs with GSH is often limited [117].

The theory of hard and soft, acid and bases (HSAB) has proven to be a useful tool to interconnect protein and DNA toxic processes [118]. Hard electrophiles will react more readily with hard nucleophilic centers in DNA, while soft electrophiles will preferably react with proteins (cysteines) (see Figure 9) [7, 106]. In contrast to DNA as the target, GSH has limited efficiency in trapping hard electrophilic molecules [119].

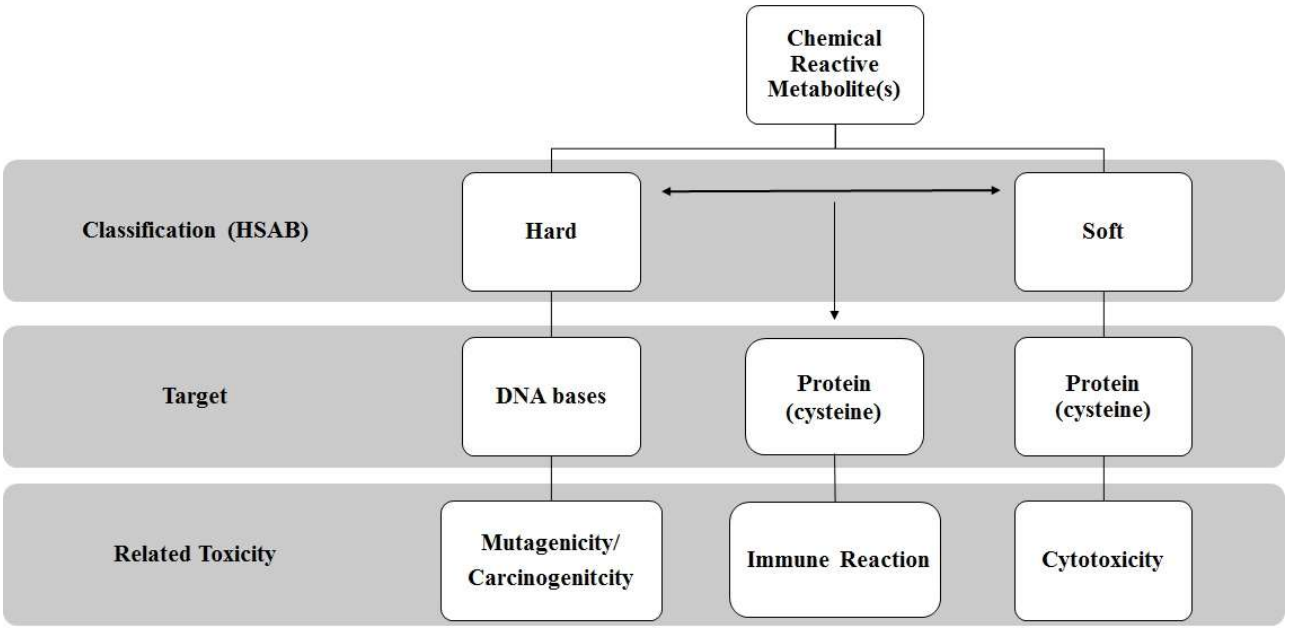

Figure 9. Application of the theory of hard and soft, acid and bases (HSAB) to interconnect protein and DNA toxic processes. Adapted from [7, 106, 118].

1.5. BIOACTIVATION AND CHEMICAL CARCINOGENESIS

1.5.1 Chemical carcinogenesis.

Cancer development or carcinogenesis is a complex multistage process. Conceptually, it can be divided into four stages: initiation, promotion, malignant conversion and progression [120].

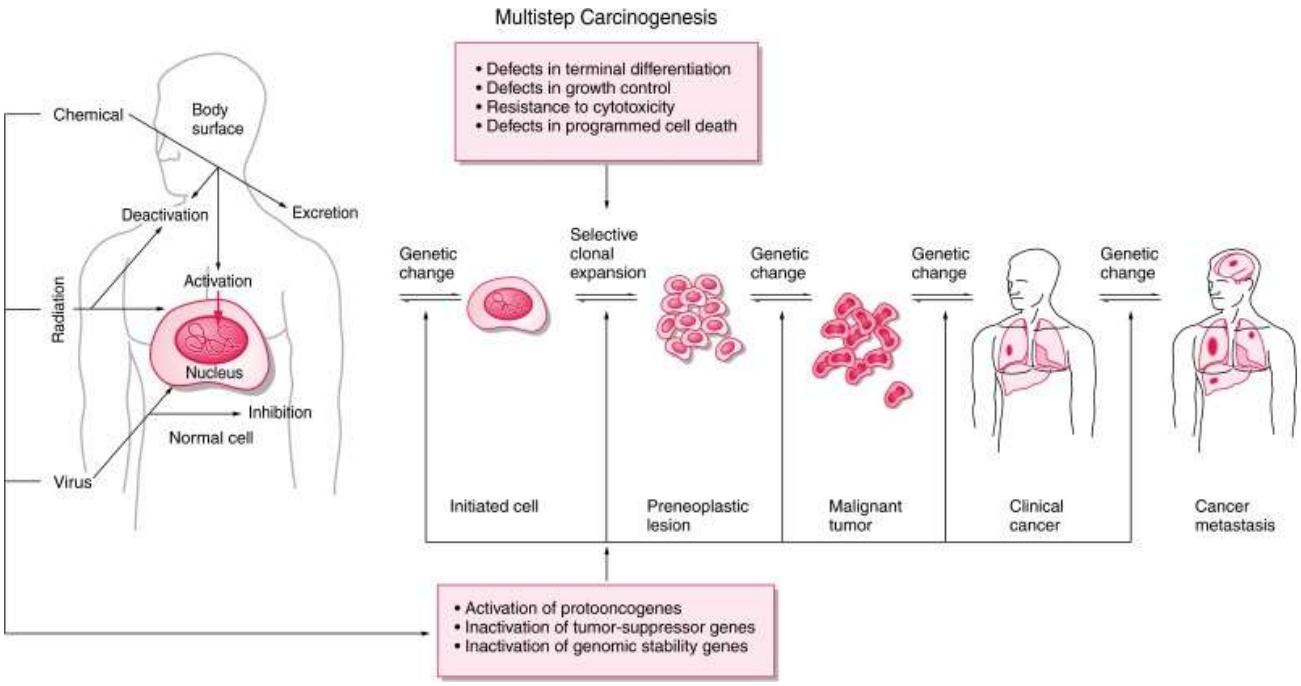

Figure 10. Scheme of multistage carcinogenesis. Multistage carcinogenesis can be conceptually separated into four stages: tumor initiation, tumor promotion, malignant conversion, and tumor progression. The activation of proto-oncogenes and inactivation of tumor suppressor genes are mutational events that result from covalent damage to DNA caused by chemical exposures. The accumulation of mutations, and not necessarily the order in which they occur, constitutes multistage carcinogenesis. Adapted from [121].

It has been broadly accepted that carcinogenesis often results from chemical exposure, sometimes in complex chemical mixtures, encountering in the environment or through our diet and lifestyle, together with biological (virus) and physical (radiation) exposures [120]. The typical example is tobacco smoke, which is related to several cancers, including lung, bladder, head and neck cancers [122, 123]. Although the majority of chemical carcinogens do not react directly with cellular components, they are often bioactivated to mutagenic and carcinogenic metabolites by drug metabolizing enzymes, such as CYPs. Thus, enzymatic biotransformation plays a crucial role in the carcinogenic activity and organ specificity of chemical carcinogens, with the predominant involvement of CYPs [124, 125].

Recent advances in epigenetics, have shown that these mechanisms are critical for the stable propagation of gene activity states between generations of cells and therefore the epigenome governs the establishment and maintenance of cell identity [126]. The failure in correct preservation of heritable epigenetic marks may result in inappropriate activation/inhibition of various signaling pathways leading to disease states such as cancer [127, 128]. It has been shown that human cancer cells harbor epigenetic abnormalities, in addition to genetic alterations [127, 129]. However, the genetic origin of cancer is broadly accepted, some studies suggest that epigenetic modifications may be a key factor in some forms of cancer [130].

1.5.2 Bioactivation, DNA adduct formation, DNA repair and mutagenesis.

Despite the fact that some compounds can act as direct mutagens, such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a large proportion of mutagens are chemically inert and require metabolic activation to induce their detrimental effects [131, 132].

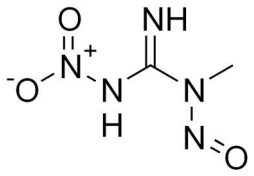

Figure 11. Chemical structure of MNNG, a direct acting DNA alkylating agent. MNNG reacts with DNA to form adducts at the O^6 of guanine and O^4 of thymine of DNA [133].

Chemical modifications of DNA, known as DNA adducts, e.g. induced by the exposure to genotoxins and/or their CRMs, have been widely investigated and play a key role in chemically induced carcinogenesis [134-136]. If not repaired, DNA adducts can

lead to mutations during cell division and thus ultimately disrupt the functioning of the systems regulating cell differentiation and proliferation [137]. However, there is a remarkable variety of DNA repair systems, which eliminate these alterations in DNA so that the ultimate mutation does not always occur. In fact, nearly all DNA adducts and DNA damage are repaired, with the rare exceptions leading to mutagenesis [131].

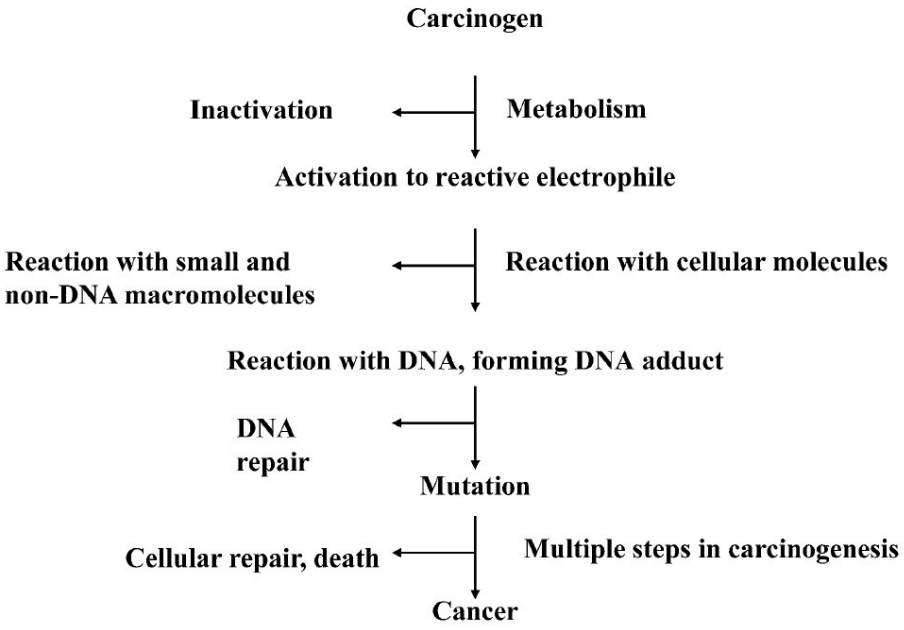

Figure 12. Competing metabolic activation and inactivation processes for DNA reactive carcinogens. Additionally, metabolic activation must occur in a pluripotential (tissue stem) cell, and the cell must replicate to fix the DNA alteration (DNA repair) produced by a DNA adduct as a permanent mutation. Adapted from [131].

Bioactivation of xenobiotics has been a pillar of carcinogenesis ever since the publications by the Millers [138] in the 1970s. These authors were able to demonstrate that an aromatic amide, 2-acetylaminofluorene (2-AAF), was metabolically activated to a reactive electrophile, *N*-hydroxy-2-acetylaminofluorene, which reacts with DNA to form DNA adducts, ultimately leading to mutations. Since then, a diversity of metabolic processes has been identified in the bioactivation of a variety of classes of chemical carcinogens, primarily involving CYP enzymes. Numerous other enzyme systems have

also been identified in the specific activation of xenobiotics (see Figure 11) [132, 139]. Variability in metabolic capacity can modulate the levels of DNA adducts ultimately formed, depending on the route of exposure and the tissues involved. Amongst others, polymorphisms in drug metabolizing enzymes (such as CYPs) and DNA repair efficiency, have been shown to alter the potential for DNA damage [140].

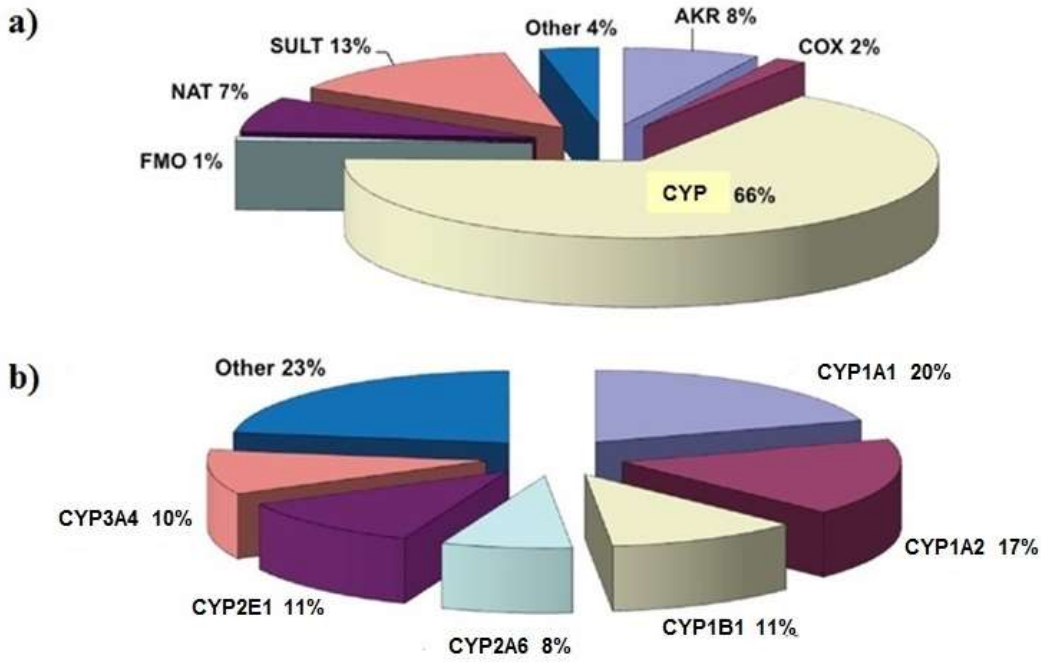

Figure 13. Enzyme contributions to bioactivation of procarcinogens. **a)** Fractions of bioactivation reactions ascribed to different groups of enzymes. A number of 713 reactions were accounted for this analysis. **b)** Fractions of CYP-mediated bioactivation reactions attributed to individual human CYPs (473 reactions were considered). Adapted from [132].

1.6. ADVERSE DRUG REACTIONS

Adverse drug reactions (ADRs) are a major complication of drug development and therapy. It has been estimated that ADRs cost to the American society about 100 billion US$ and cause more than 100,000 deaths per year [35]. Human adverse events, animal toxicity and clinical safety represent together about 56% of the attrition reasons

for withdrawal of drug candidates during drug development [141, 142]. Also because of the increasing of commercial consequences of drug withdrawals and/or black box warnings due to safety issues, the pharmaceutical industry faces an unprecedented challenge of providing patients with more effective and safer products [143].

The mechanisms underlying ADRs may be categorized into five types [9]: 1) on-target or mechanism-related; 2) hypersensitivity and related immunological responses 3) bio-activation to toxic metabolites, 4) off-target and 5) idiosyncratic adverse drug reactions (IADRs). About 27% of the causes of attrition due to toxicity are related to biotransformation and about 28% are target-based (off- and on-) [142]. Moreover, drug accumulation, excessive dose and/or biotransformation to CRMs have been associated in several off-target and IADRs [113]. Although the exact mechanisms by which IADRs arise is not fully understood, retrospective studies of failed drugs indicate that drug metabolism and the generation of CRMs play an important role [110].

1.7. *IN VITRO* ASSAYS FOR GENOTOXICITY STUDIES

DNA reactivity is an unwanted property of a drug or drug metabolites, i.e. leading to increased risk of cancer and reproductive toxicity. The general strategy to minimize this property is to eliminate genotoxic risks as early as possible, normally in early stages of industrial chemicals production or in the hit-to-lead/lead optimization phase of drug development [144]. Simultaneously, there is a need for simple and rapid methods for evaluating the potential (geno)toxic effects of diverse environmental samples [145].

Currently, genotoxicity testing guidelines for pharmaceutical industry incorporate a battery of *in vitro* and *in vivo* tests to identify hazards, measuring different types of genetic damage in a diversity of cell models to increase the probability of detecting DNA-reactive compounds [146]. Unfortunately, the screening of thousands of compounds using

whole animal models is neither economically viable nor ethically acceptable, posing high-throughput screening (HTS) assays as an alternative for handling larger flows of new chemicals or drug candidates in a systematic and time-efficient manner [147].

In such *in vitro* tests, the usually examined end points include: i) induction of point mutations in bacteria; ii) induction of point mutations and chromosomal aberrations in mammalian cells; and iii) production of strand breaks and covalent modifications to DNA [146]. However, differences that may arise from biotransformation, due to the conditions of standard *in vitro* test systems (normally using induced rat liver extracts or other animal species), have a major influence when establishing the relevance for human risk assessment [5]. Moreover, inter-species differences in drug metabolism are consistently observed and often lead to complications in data extrapolation to the humans from animal safety data [13]. This is of particular importance when the critical toxic entity is not the chemical itself but a CRM, whether it applies to pharmaceuticals, pesticides or industrial chemicals [5].

In vitro cell-models for genotoxicity testing are of particular interest due to their relative simplicity in manipulation, the collection of data is in short term and are in line with the societal concern of laboratory animal use. Currently, the role of metabolism in drug and industrial chemical development, when using standardized mutagenicity *in vitro* assays, is achieved using induced rat liver extracts as exogenous metabolic system for detecting pro-mutagens [148, 149]. In fact, a major limitation in detecting genotoxicity of human CRMs by standard *in vitro* tests resides in the fact that most of these assays rely on non-human (usually rodent derived) metabolic systems, to mimic human metabolism [5]. Moreover, when using this approach, the metabolites (including CRMs) are often generated outside the target cells, with less probability to interact with the DNA target.

Therefore, there still is a collective need for sensitive and highly efficient cell-systems for *in vitro* genotoxicity studies, incorporating (polymorphic) human biotransformation and allowing more efficient screening of increasing numbers of compounds with an increased reliability and predictability. Of special need in this context, are assays with the appropriate properties for high-throughput screening (HTS). Accordingly, several *in vitro* assays, using bacteria, yeast or human cell-lines were developed for HTS purposes, making use of reporters such as GFP and/or luciferase, which permit to obtain results in a timely manner. Amongst others, these include SOS-lux [150], VITOTOX™ [151], GreenSreen® [152], BlueScreen™ [153] and p53 CALLUX [154] assays.

1.7.1 Bacterial genotoxicity assays for high-throughput screening (HTS)

Lately, the progress in chemical technologies has widely expanded the numbers of leads for new industrial chemicals and potential drug candidates. Furthermore, the requirement of thousands of toxicity profiles due to the implementation of the new EU chemical policy, designated REACH, reinforced the need for efficient and highly predictive HTS (geno-)toxicity models [1]. Hard electrophilic CRMs may react with DNA and might be detected in genotoxicity assays. In this context, bacterial assays (see Table II) are of special interest, presenting several characteristics that position them as highly attractive cellular systems, namely: fast to respond; easily obtained large and homogeneous population of live cells; low costs; conservation for longer periods of time; available at short notice; and lack of background in biotransformation [2, 145].

Table II. Examples of bacterial genotoxicity assays. Adapted from [155].

Test method	Bacterium	SOS gene	Index	References
Ames test	*S typhimurium*	*mucAB*	his	[156]
WP2 test	*E. coli*	*mucAB*	trp	[157]
Induct test	*E. coli*	Prophage	λ phage	[158]
SOS chromotest	*E. coli*	*sfiA::lacZ*	β-gal	[159]
***umu* test**	*S typhimurium*	*umuC::lacZ*	β-gal	[160]
SOS *lux* test	*E. coli*	*luxCPABFE*	lux	[150]
VITOTOX® test	*S typhimurium*	*recN2-4::lux*	lux	[151]
Geno-Tox test	*S typhimurium*	*Cda::lux*	lux	[161]

S. typhimurium, *Salmonella typhimurium*; *E. coli*, *Escherichia coli*; his, histidine; trp, tryptophane; β-gal, β-galactosidase; lux, luciferase.

Apart from the standard, well-established Ames test, several other bacterial tests based on the induction DNA-repair (SOS)-response for detecting mutagenicity [162], namely the *umu*-test [160] and the SOS chromotest [159] were developed to rapidly assess the potential properties for carcinogenicity of drug candidates and other xenobiotics. Still, currently these assays can hardly be considered as HTS assays, since they are time consuming and/or demand many manipulation steps. More recently, several other bacterial test systems, also based on the induction DNA-repair (SOS)-response, were developed for HTS purposes and making use of reporters such as GFP and/or luciferase, which permit to obtain results faster. These include SOS-lux [150] and VITOTOX™ [151], assays. In all cases, rodent liver extracts are used has exogenous bioactivation system for detecting pro-mutagens.

1.8. AIMS AND OUTLINE OF THIS THESIS

1.8.1 Aims of this thesis

When the studies described in this thesis started, there was still an ever increasing need for informative and highly efficient systems for *in vitro* studies of xenobiotics in toxicology, and in particular for cell-systems for genotoxicity studies. Moreover, in this context, assays with the appropriate properties for HTS applications are still needed.

The *primary aim* of the research of this thesis was to develop improved and sensitive bacterial genotoxicity test systems, which were (i) metabolically competent in terms of human biotransformation and (ii) suitable for HTS testing applications, allowing efficient screening, both in terms of time and costs. For reasons of relevance and applicability in this context, the research was first focused on one of the major human biotransformation enzyme systems, namely CYP. *S. typhimurium* and E. *coli* strains were used, since, bacterial assays present several advantages, such as: fast response; easy to obtain large and homogeneous populations of live cells; low costs; long conservation of strains; available at short notice; and lack of background in biotransformation. The newly developed bacterial test systems should also be applicable in genotoxicity and mechanistic studies using other, major human CYPs as well as allelic CYP-variants.

The *secondary aim* of the research was to investigate the significance of alleles of CYPs, representing non-synonymous polymorphic forms, and to characterize: (i) their activity with a diverse group of substrates and (ii) the role of the accessory redox partner cytochrome b_5 in their activity. In this case, for mechanistic studies, *E. coli* strains were chosen, since they are easier to manipulate genetically and have been broadly been used to express CYPs. As for the genotoxicity assay *S. typhimurium* strains were preferred, because aromatic amines require CYP1A2-mediated oxidation and subsequent O-acetyl or O-sulfate conjugation to generate CRMs which react with DNA [163]. *S. typhimurium*

tester strains of the Ames test (namely, TA1535) are known to contain efficient bacterial O-acetyltransferases [164].

In this thesis, different bacterial backgrounds, namely TA1535 and TA100, two *S. typhimurium* strains used in the Ames test, and strains FP401 and PD301, two *E. coli* strains developed in our laboratory (Lisbon), were used for development of the genotoxicity system. Human CYP1A2 and several CYP1A2 polymorphic variants were chosen as model enzymes for proof of concept, since these CYP enzymes in particular constitute important enzymes in CRMs formation. Notably CYP1A2 catalyzes the bioactivation of approximately 17% of human pre-carcinogens [132], such as aromatic and heterocyclic amines [165] and mediates the metabolism of about 15% of clinical drugs [61].

1.8.2 Outline of this thesis

Part I: Introduction

In the present chapter, **chapter 1**, a general introduction to the work presented in this thesis is provided. Xenobiotic biotransformation and the role of CRMs in genotoxicity and ADRs are discussed. In this context, the CYP superfamily, including the electron donor accessory enzymes (b_5 and CYPOR), is presented with special focus on CYP1A2 isoform. Chemical carcinogenesis and mutagenesis are introduced, and *in vitro* assays for genotoxicity detection are discussed, with emphasis on bacterial systems.

Part II: Characterization of Human Cytochrome P450 1A2 Polymorphic Variants

In **chapter 2**, the construction and characterization of 8 non-synonymous polymorphic forms of human CYP1A2, namely T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H is described. The cDNAs of these variants plus WT form, are

co-expressed with human CYPOR in *Escherichia coli* BTC [166] strains. The activity of the CYP1A2 variants is screened using 8 different substrates: 3 fluorogenic probes, 2 therapeutic drugs and 3 pro-mutagens. The resulting data set, from 16 different activity parameters measured, is thoroughly analyzed using multivariate analysis. Results are then interpreted using the human CYP1A2 crystal structure. **Chapter 3**, describes the expression and characterization of the same 8 polymorphic forms of human CYP1A2 plus WT, but now co-expressed with b_5 and CYPOR. Again, 16 activity parameters for the 8 substrates are screened, and the results merged with the ones obtained in the previous chapter. This combined data set is then analyzed through multivariate analysis and the results rationalized using human CYP1A2 crystal structure.

Part III: Development of Bacterial HTS-Systems for Detection of Genotoxic Electrophilic Reactive Metabolites

In **chapter 4**, the engineering of bacterial genotoxicity systems suitable for HTS purposes is presented. Two different types of GFP plasmid reporters, specifically for genotoxicity and cytotoxicity, were constructed and expressed into *Escherichia coli* and *Salmonella typhimurium* strains. Initially, four different bacterial backgrounds are tested, namely, TA1535 and TA100 [167], two *S. typhimurium* strains used in the Ames test, and strains FP401[168] and PD301[166], two *E. coli* strains developed in our laboratory. All strains are tested with three direct-acting mutagens. Subsequently, the two most effective bacterial systems, based on strains TA1535 and PD301, are adapted to co-express human CYP1A2 and CYPOR, along with the GFP reporters. These strains are then tested with three known pro-mutagens that form DNA-reactive metabolites through CYP1A2-mediated bioactivation.

Chapter 5, describes the co-expression of CYPOR with the same 8 polymorphic forms of human CYP1A2 plus WT, used in **chapters 2** and **3**. In this case, the system based on *S. typhimurium* TA1535 strains is applied and tested with the three CYP1A2-dependent pro-mutagens. The resulting data set is analyzed using multivariate analysis and results compared with the results obtained in the previous chapters.

Part IV: Conclusions

In **chapter 6**, a summary and the conclusions regarding the research described in this thesis are given as well as future perspectives for further developments of the bacterial genotoxicity systems presented.

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PART II

CHARACTERIZATION OF HUMAN CYTOCHROME P450 1A2 POLYMORPHIC VARIANTS

CHAPTER 2

Functional characterization of eight human cytochrome P450 1A2 gene variants by recombinant protein expression

Adapted from: Palma, B.B.; Silva e Sousa, M.; Vosmeer, C.R.; Lastdrager, J.; Rueff, J.; Vermeulen, N.P.E.; Kranendonk, M.; "*Functional Characterization of Eight Human Cytochrome P450 1A2 gene variants by recombinant protein expression*" Pharmacogenomics J. 2010 Dec; 10(6):478-88.

ABSTRACT

Inter-individual variability in cytochrome P450 (CYP)-mediated xenobiotic metabolism is extensive. CYP1A2 is involved in the metabolism of drugs and in the bioactivation of carcinogens. The objective of this study was to functionally characterize functionally eight polymorphic forms of human CYP1A2, namely T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H. The cDNAs of these variants were constructed and coexpressed in *Escherichia coli* with human NADPH cytochrome P450 oxidoreductase (CYPOR). All variants showed similar levels of apoprotein and holoprotein expression, except for I386F and R456H, which showed only apoprotein, and both were functionally inactive. The activity of CYP1A2 variants was investigated using 8 substrates, measuring 16 different activity parameters. The resulting heterogeneous activity data set was analyzed together with CYP1A2 wild-type (WT) form, applying multivariate analysis. This analysis indicated that variant G299S is substantially altered in catalytic properties in comparison to WT, whereas variant T83M is slightly but significantly different from the WT. Among the CYP1A2 variants, out of the heterogeneous set of eight substrates, carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was the most discriminative compound. In addition, R456 could be identified as an important residue for proper heme binding and stabilization.

2.1. INTRODUCTION

Inter-individual variability in xenobiotic and drug metabolism is extensive. Plasma levels of a drug can vary more than 1000-fold between individuals upon the same drug dosage [1]. These variations can be caused by induction or inhibition and genetic polymorphisms of metabolizing enzymes, or from physiological, pathophysiological or environmental factors [2]. In fact, genetic factors are considered to be responsible for 20-40% of the differences between individuals in the response and susceptibility to drugs and xenobiotics [3]. Variability among individuals in drug metabolism can result in reduced drug efficacies or undesirable toxic side effects, which are phenomena that may compromise drug therapy and development.

Cytochrome P450s (CYPs) are responsible for the metabolism of a wide variety of clinically, physiologically and toxicologically important compounds [4]. CYP families 1, 2 and 3 are responsible for 70-80 % of all phase I metabolism of clinically used drugs [5] and are involved in the biotransformation of a large number of xenobiotics. Biotransformation can lead to bioactivation of pre-carcinogens and drugs, which can result in carcinogenic or other toxic effects. The majority of CYP-mediated xenobiotic metabolism is carried out by polymorphic enzymes [6]. Human CYP polymorphisms include mutations both in non-coding regions (promoter, intron) and protein-coding sequences. Mutations in non-coding regions may lead to altered expression levels, whereas those in protein-coding sequences may change the structure and catalytic properties of the enzyme [7]. Many drugs that can cause adverse reactions are metabolized in processes catalyzed by polymorphic CYP enzymes. Therefore, the understanding of the nature of CYP polymorphisms is fundamental for the comprehension of inter-individual differences in chemical exposure, adverse drug effects and toxicity in general [8].

The human CYP1A family consists of CYP1A1 and CYP1A2. Their genes are located on chromosome 15 and are oriented head-to-head, 23.3 kb apart [9]. The spacer sequence may contain distinct regulatory regions of each gene or, alternatively, the regulatory regions of both genes [10]. CYP1A1 is mainly expressed in extrahepatic tissues, whereas CYP1A2 is almost exclusively expressed in the liver, representing about ~15 % of its total CYP content [11]. CYP1A enzymes catalyze the bioactivation of pre-carcinogens, such as polycyclic aromatic hydrocarbons (CYP1A1) as well as aromatic and heterocyclic amines (CYP1A2) [12]. CYP1A2 is also responsible for the metabolism of drugs, such as phenacetin [13], clozapine [14, 15], naproxen [16], propranolol [17] and tizanidine [18].

The CYP1A2 gene consists of seven exons, including non-coding exon 1 [19]. Inter-individual differences in CYP1A2 activity are well known: up to 60-fold variations have been reported [11, 20]. In addition, ~15- and 40-fold variations in mRNA and protein expression levels have been observed in the human liver [19, 21]. To date, 36 CYP1A2 haplotypes have been published on the Human Cytochrome P450 Allele Nomenclature Committee home page (*http://www.cypalleles.ki.se/cyp1a2.htm*). Of these, 21 are in the non-coding region and 15 are in the coding region, all causing amino acid changes of the CYP1A2 protein. Apart from studies that characterized gene polymorphisms in the non-coding region, few reports described non-synonymous protein polymorphisms. Mostly, these concern the identification of genetic polymorphisms and their frequencies, but not their effects on protein level and activities. Murayama *et al* [22], Saito *et al* [23] and Zhou *et al* [7] characterized most of the non-synonymous polymorphisms with a limited number of substrates. The primary objective of this study is to investigate polymorphic forms, representing the majority of alleles with the non-synonymous polymorphic CYP1A2 protein, and to characterize their activity with a diverse group of substrates.

On the basis of a crystal structure published by Sansen *et* al [24] of CYP1A2 wild-type (WT) [CYP1A2*1] we selected mutants that seem to be localized in the vicinity of the active center, heme, entrance/exit channel or the NADPH-cytochrome P450 oxidoreductase (CYPOR) interaction zone. As such, these polymorphic forms concern T83M [CYP1A2*9], S212C [CYP1A2*12], S298R [no allele designation], G299S [CYP1A2*13], I314V [no allele designation], I386F [CYP1A2*4], C406Y [CYP1A2*5] and R456H [CYP1A2*8]. For this purpose, the cDNA of the WT allele, present in a bacterial expression vector [25], was adapted through genetic engineering to generate the eight different CYP1A2 forms. CYP1A2 alleles were coexpressed with human CYPOR in a bacterial cell model [26], and the activity of the eight allelic variants and WT was investigated. The enzyme activity of these variants was analyzed together with WT and scrutinized through multivariate analysis. Results of this analysis were interpreted using the CYP1A2 crystal structure [24].

2.2. MATERIALS AND METHODS

2.2.1. Reagents

L-arginine, ampicillin, kanamycin sulfate, chloramphenicol, cytochrome *c*, isopropyl β-D-thiogalactoside (dioxane-free) (IPTG), thiamine, 2-aminoanthracene (2AA), glucose-6-phosphate, NADPH, ethoxyresorufin (ER), methoxyresorufin (MR), resorufin, phenylmethanesulfonyl fluoride (PMSF) and α-naphthoflavone (α-NF) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2-Amino-3-methylimidazo(4,5-*f*)quinoline (IQ) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). 3-cyano-7-ethoxycoumarin (CEC) and 3-cyano-7-hydroxycoumarin (CHC) were obtained from BD Biosciences (Bedford, MA, USA). Phenacetin was

obtained from Brocades-ACF (Maarssen, The Netherlands) and clozapine from Duchefa Farma bv. (Haarlem, The Netherlands). Bacto agar, bacto peptone, bacto tryptone and bacto yeast extract were obtained from Difco (Detroit, MI, USA). All other chemicals were of the highest quality.

2.2.2. Site-directed mutagenesis

Site directed mutagenesis was performed with the "Quickchange XL" (Cedar Creek, TX, USA) and applied according to the manufactures instructions. All variants, except I386F, were constructed using plasmid pCWh1A2 [26] as DNA template, encoding human CYP1A2*1 (isoform 2) [NCBI NM_000761] with N-terminus modified as described by Fisher *et al* [25]. In the case of the variant I386F, the template was pUC18_h1A2. This plasmid was constructed by cloning the cDNA of CYP1A2 from pCWh1A2 as an NdeI-XbaI fragment in pUC18. Forward and reverse mutagenesis primers for the creation of the desired mutation for each variant are presented in Supplementary Information Table I.

The introduced mutation for each variant was confirmed by sequencing the full extent of the CYP1A2 cDNA. The pCWh1A2 plasmid has a considerable size (6.9 Kb), and it is known that, for a site-directed mutagenesis procedure, large plasmids have a reduced but significant risk for incorporation of undesired mutations. Therefore, mutated cDNAs were re-cloned into the pCW vector to ensure that plasmid sequences remained unaltered. For this, the restriction sites NdeI and XbaI were used, and the cDNA was subsequently resequenced.

2.2.3. Bacterial coexpression of CYP1A2 variants with CYPOR and membrane preparation

The expression of the different variants of CYP1A2 with CYPOR was obtained as described previously, using the bi-plasmid coexpression system [26]. Briefly, each strain was cultured in the TB (Terrific Broth) medium supplemented with peptone (2 g l^{-1}), thiamine (1 μg ml^{-1}), ampicillin (50 μg ml^{-1}), kanamycin (15 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), trace elements solution [27] (4 μl ml^{-1}), and 0.2 mM isopropyl β-D-thiogalactoside (dioxane-free) (final concentrations). Cultures were started with 250 μL of –80 ºC glycerol stocks, and cells were grown for 16 h at 28 ºC with moderate agitation.

Membrane preparations were isolated as follows. Cultures were harvested at 2772 × *g* for 20 min at 4 °C. The pellet was resuspended in TS (Tris-sucrose) buffer (75 mM TrisHCl, 250 mM sucrose, pH 7.8). Lysozyme was added to a final concentration of 0.5 mg/ml, and cells were incubated on a roller bench for 30 min at 4 °C. Subsequently, EDTA (0.5 mM) and the protease inhibitor phenylmethanesulfonyl fluoride (0.5 mM) were added. Cell lyses was performed by freezing (–80 ºC) and thawing (1 cycle) and by subsequent several short rounds (60 s) of low-intensity sonication, interspersed with 30 s of ice-bath submersion, such that the temperature of the preparation never exceeded 8 ºC. The suspension was centrifuged at 2772 × *g*, for 10 min at 4 °C to eliminate unbroken cells. Membranes were pelleted by ultracentrifugation of supernatant at 100 000 × *g*, at 4 °C for 60 min. Membranes were resuspended in TGE buffer (75 mM TrisHCl. 10 % (v/v) glycerol, 25 mM EDTA, pH 7.5) using a Dounce homogenizer, Jencons PLS (East Sussex, UK) and stored at –80 °C. In the case of phenacetin and clozapine experiments, membranes were obtained from cells, which had been lysed by French Press (3 cycles at 1000 psi on ice) and a subsequent sonication treatment (2 cycles of 2 min). Protein concentrations were determined using the method described by Bradford, following the

manufacturer's protocol from Bio-Rad (San Francisco, CA, USA), using bovine serum albumin as the standard.

The CYP content of whole cells and membrane preparations, was determined using the CO-difference spectra technique as described previously [28]. The CYPOR content of membrane preparations was determined using the NADPH-cytochrome *c* reduction assay as described previously [29].

2.2.4. Western blot analysis

Membrane proteins (10 μg) were separated by SDS-PAGE electrophoresis (10 %) and electrotransferred to nitrocellulose membranes Hybond-ECL from GE Healthcare (Uppsala, Sweden). Immunoblotting analyses were carried out with the enhanced chemiluminescent ECL western blotting detection kit from GE Healthcare, using as primary antibody a polyclonal antibody from rabbit serum raised against recombinant human CYP1A2 from CYPEX (Dundee, UK).

2.2.5. 7-methoxy- and 7-ethoxy-resorufin *O*-dealkylation

7-Methoxy- (MROD) and 7-ethoxy-resorufin (EROD) *O*-dealkylation activities was performed in a 96-well microplate format, as described by Kranendonk *et al* [29] using a Zenyth 3100 microplate reader from Anthos (Imola, Italy). All measurements were carried out in triplicate and were performed at least three times in separate experiments. Stock solutions of MR and ER were prepared in dimethyl sulfoxideand were varied over 8 concentrations (MR: ≤ 2 μM; ER: ≤ 5 μM), maintaining a constant final solvent concentration of 0.2% (v/v) throughout the experiment, thus preventing interference with CYP1A2 activity [30]. The reactions were followed for the first 15 min. Velocity data were plotted according to the Michaelis–Menten equation, and kinetic

parameters (V_{max} and K_M) were calculated using GraphPad Prism from GraphPad software (La Jolla, CA, USA) applying one binding site analysis (hyperbola).

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2.2.6. 3-Cyano-7-ethoxycoumarin *O*-dealkylation

The 3-cyano-7-ethoxycoumarin *O*-dealkylation (CECOD) activity was measured as the rate of the increase in CHC (excision: 409 nm; emission: 460 nm). All measurements were carried out in triplicate and were performed at least three times in separate experiments, in a 96-well microplate format using a Zenyth 3100 microplate reader. Rates (μM CHC formed per min) could be calculated using a fluorescence standard curve of the product CHC. The reaction was performed with membranes (1 pmol of CYP1A2 per well) under the same conditions as for EROD and MROD. Stock solutions of CEC were prepared in acetonitrile and were varied over eight concentrations (≤ 75 μM), maintaining a constant final solvent concentration of 0.2% (v/v) throughout the experiment. The reactions were followed for the first 15 min. Velocity data were plotted according to the Michaelis–Menten equation, and kinetic parameters (V_{max} and K_M) were calculated using GraphPad prism software, applying one binding site analysis (hyperbola).

2.2.7. Inhibition studies

IC_{50} determinations for α-NF were performed for MROD, EROD and CECOD as described above. In all, 12 different α-NF concentrations (≤ 5 μM) were used for each substrate. Substrate concentrations were 1.0, 2.5, and 25.0 μM for MR, ER and CEC, respectively.

The activity for each inhibitor concentration was determined at least in triplicate. IC_{50} was calculated with GraphPad prism software using sigmoidal dose-response analysis.

2.2.8. Clozapine *N*-demethylation

Clozapine *N*-demethylation activity was measured as the rate of the increase in demethylated clozapine absorbance at 254 nm using high-performance liquid chromatography (HPLC) coupled with a Separations 759A absorbance detector from Applied Biosystems (Foster City, CA, USA). All measurements were performed in triplicate and at least three times in separate experiments. Rates (pmol demethylated clozapine formed per min) were estimated using a standard curve of the substrate (clozapine), assuming that the extinction coefficients of the substrate and metabolite (demethylated clozapine) are comparable [31].

To verify Michaelis-Menten characteristics (that is, time, substrate and enzyme dependence) of the metabolism of clozapine by CYP1A2, a full kinetic analysis with WT was performed. For this purpose, 100 nM of CYP1A2 was used per incubation and a gradient of 7 clozapine concentrations (≤ 500 μM). Analysis of clozapine demethylation by the 8 polymorphic CYP1A2 forms was performed with 100 nM of enzyme and 50 and 250 μM of clozapine. All incubations were performed in a final volume of 300 μL, and incubated for 60 min at 37 ºC. Reactions were initiated by the addition of 3.3 mM NADPH and terminated with 30 μL of 10% (v/v) $HClO_4$. The precipitated protein was removed by centrifugation (for 15 min at 4000 × *g*) and the supernatant was analyzed by HPLC. Metabolites were separated using a C18 Luna 5-U 100A column from Phenomenex (Torrance, CA, USA) at a flow-rate of 0.6 ml/min. A gradient program was applied, using two mobile-phase solutions: A contained 99% H_2O, 1% acetonitrile and

0.02% formic acid and B contained 99% acetonitrile, 1% H_2O and 0.02% formic acid. The separation was initiated with 100% A, and maintained for 5 min. Subsequently, solution B was increased linearly to reach 100 % after 30 min and the percentage of solution B was inversed to 0% in 5 min.

2.2.9. Phenacetin *O*-deethylation

Phenacetin *O*-deethylation activity was measured as the rate of the increase in acetaminophen absorbance at 254 nm by HPLC analysis using a Separations 759A absorbance detector. All measurements were performed in triplicate and at least three times in separate experiments. Rates (pmol acetaminophen formed per min) could be calculated by using a standard curve of the product acetaminophen.

In order to verify Michaelis-Menten characteristics (time, substrate and enzyme dependence) of the metabolism of phenacetin by CYP1A2, a full kinetic analysis with WT was performed. For this purpose, 100 nM of CYP1A2 was used per incubation and a gradient of 7 phenacetin concentrations (≤ 400 μM). Analysis of phenacetin deethylation by the 8 polymorphic CYP1A2 forms was performed with 100 nM of enzyme and 20 and 200 μM phenacetin. Incubations and HPLC analysis were performed as for clozapine.

2.2.10. Bioactivation assays

The bioactivation/mutagenicity assays were performed using the liquid pre-incubation assay technique, as described previously [32]. Experiments were performed in triplicate and at least three times in separate experiments. Revertant colonies were counted after 48h of incubation at 37 ºC. Plates were digitally photographed, and the number of L-arginine prototrophic revertant colonies per plate were determined using

Labworks Software (version 4.6) from UVP, Inc. (Upland, CA, USA). Mutagenic activities (in revertants per nmol or revertants per μmol) were determined from the slope of the linear portion of the dose-response curve.

2.2.11. Multivariate statistical analysis

The normalization procedure was based on the variance in the data set. Both column and row variances were applied. This type of double normalization is appropriate when different enzyme preparations are being compared (that is, using partially purified or subcellular fractions) and when different types of substrates and detection methods are used [33]. Data pre-processing was carried out by dividing the values of the initial data set by the row-column double variance:

$$X_{norm} = (X_{obs} / Var_{col}) / Var_{row}$$

Differences between polymorphic variants and WT were analyzed globally by principal component analysis (PCA)[34] and by multidimensional scaling (MDS)[35].

2.3. RESULTS

2.3.1. Site-directed mutagenesis, mutant enzyme expression and characterization

The eight polymorphic variants of human CYP1A2 were constructed and expressed in *Escherichia coli*. A suitable *E. coli* host, the BTC strain, was applied for adequate coexpression of the CYP1A2 allelic variants with human CYPOR, using a bi-plasmid system [26]. CYPOR expression levels were determined in the membrane protein fraction and were comparable among all strains (7.9-11.4 pmol/mg membrane protein). The CYPOR/CYP ratios for the different variants were found in the range of the normal

values as observed in human liver microsomes (0.08-0.5) [36] except for variants I386F and R456H, which had no detectable CYP holoenzyme.

Immunoblot analysis of membrane preparations indicated that CYP-protein expression levels were similar for WT and all eight constructed variants (Supplementary Figure S1). The CO-difference spectra of all recombinant CYPs expressed in *E. coli* BTC were also determined to quantify the CYP1A2 holoenzyme content (Supplementary Figure S2). Holoprotein levels for most variants were comparable with or slightly lower than WT, except for variants I386F and R456H. I386F showed a strongly reduced 450-nm peak and a large 420 nm peak in bacterial cells (Supplementary Figure S2), and a total absence of the 450-nm peak in membrane fractions (data not shown). This indicates instability of the I386F holoprotein, probably due to inefficient heme incorporation and/or anchoring as a stable apoprotein has been demonstrated by immunodetection (Supplementary Figure S1). For R456H, no 450-nm peak was detectable in either whole cells or membrane preparations, indicating the absence of holoprotein.

2.3.2. WT and mutant CYP1A2 activities

To determine enzyme activities, all allelic variants were studied using eight structural different substrates and were compared with WT (Figure 1). Three fluorogenic CYP1A2 substrates (ER, MR and CEC), and two therapeutic drugs (clozapine and phenacetin) were used. Furthermore, the bioactivation of three CYP1A2 dependent pre-mutagens (NNK, 2AA and IQ) was measured. All results are summarized in Table 1.

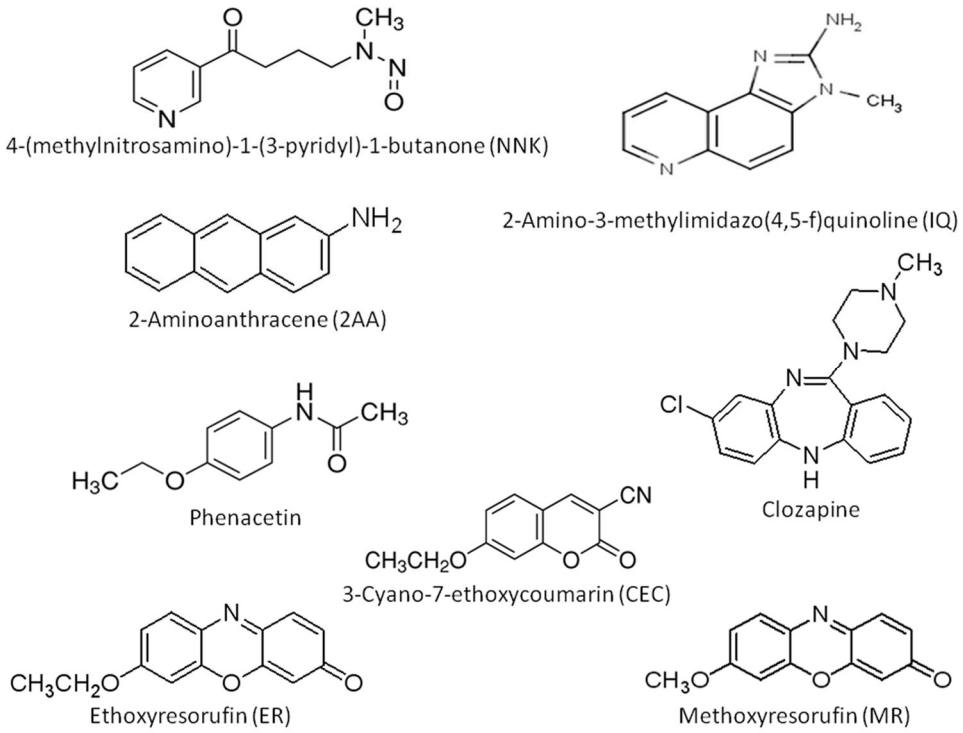

Figure 1. Chemical structures of used substrates demonstrating the full structure space of CYP1A2 compounds applied in this study.

2.3.2.1. Kinetic studies with fluorogenic substrates

The polymorphic variants and WT were tested with three typical CYP1A2 fluorogenic substrates (ER, MR and CEC). The results of the Michaelis-Menten kinetic studies (yielding V_{max} and K_M values) and the inhibitory potency (IC_{50}) of α-NF, a specific CYP1A inhibitor, are presented in Table 1. For I386F and R456H, lacking detectable CO-difference spectra, no activity was detected for any of the three substrates, not even at a double membrane sample concentration and at the highest substrate concentration. Therefore, these two variants were excluded from the remaining analysis.

Although, K_{cat} (V_{max}/K_M) is considered to be a valid kinetic descriptor, subtle but significant differences in K_M or V_{max} can be obscured. As such, K_M and V_{max} were brought forward separately. Variants T83M and I314V showed a small increase in V_{max} compared with WT, for the three substrates. In addition, the affinity for these substrates was altered,

except for CEC, with K_M values being slightly higher for MR and ER. Variant S298R demonstrated a small decrease in V_{max} both for MROD and EROD, whereas the CECOD activity was comparable with WT. Variants G299S and C406Y showed higher values for V_{max} of MROD, but not for EROD and CECOD. Affinity of variants S298R and G299S for all substrates was found similar to WT, with K_M values being comparable with WT. In the case of C406Y, the observed substrate affinities were comparable with WT for CECOD but not for MROD and EROD, with K_M values being higher in both cases. Finally, variant S212C showed activities almost identical to WT, for the three fluorogenic substrates, all V_{max} and K_M values being comparable with WT.

2.3.2.2. Kinetic studies with clozapine and phenacetin

Both clozapine *N*-demethylation and phenacetin *O*-deethylation activity of the CYP1A2 variants were also determined. I386F and R456H showed no activity for any of the two substrates, even at the highest substrate and double membrane concentrations. Therefore, these two variants were excluded from the remainder of the analysis. On the basis of the kinetic analysis of WT, two substrate concentrations were chosen. The six remaining variants and WT were incubated with 50 and 250 μM of clozapine (Table 1 and Supplementary Figure S3), and 20 and 200 μM of phenacetin (Table 1 and Supplementary Figure S4). Control assays were performed with the highest concentration of each substrate in the presence of 1mM of the CYP1A2 inhibitor α -NF.

At 20 μM concentration of clozapine, a decreased *N*-demethylation activity for all variants was found when compared with WT, but this was not the case at a clozapine concentration of 200 μM. At a clozapine concentration of 20 μM, variants T83M, S212C, S298R, G299S, I314V and C406Y showed reaction velocities of 64, 74, 58, 72, 65 and 44% of WT, respectively. At a clozapine concentration of 200 μM, for T83M, S212C,

S298R, G299S and C406Y reaction velocities of 83, 89, 108, 112, and 80%, respectively, of the WT activity were obtained, and for the I314V variant 65%.

At low substrate concentrations (50 μM), phenacetin *O*-deethylation activity was not significantly different for variants T83M, S212C and I314V, being 79, 85 and 97%, respectively, of the WT activity, but was significantly decreased for S298R, G299S and C406Y, being 57, 64 and 48%, respectively, of the WT activity. At a phenacetin concentration of 250 μM, the lowest activity found was for variant S298R with 60% of the WT activity, whereas no significant differences in reaction velocities were observed for T83M, S212C, G299S, I314V and C406Y, being 117, 106, 88, 104 and 83%, respectively, of the WT activity. In the case of mutant C406Y, extra peaks on the HPLC chromatogram were observed, which increased along with phenacetin concentration, but disappeared when inhibited with α-NF, indicating a different metabolic profile for this variant (data not shown).

2.3.2.3 Bioactivation of pre-mutagens

Strain BTC1A2, coexpressing recombinant human CYP1A2 and human CYPOR, have previously been shown to correctly bioactivate several known CYP1A2-dependent mutagens [26]. The present polymorphic CYP1A2 variants were further characterized with this cell model. Three pre-mutagens (2AA, NNK and IQ) were used for this purpose (Table 1 and Supplementary Figures S5-S8). No mutagenic activity was observed with the CYP1A2 null strain for these three pre-mutagens.

When compared with WT, variants S212C and I314V showed a slight increase in mutagenic activity for 2AA, whereas variants T83M, S298R, G299S and C406Y showed a decrease in mutagenicity. For I314V, the results were comparable with WT. In the case of IQ, variants T83M, S298R, G299S and I314V showed a small decrease in

mutagenicity, whereas variants S212C and C406Y were comparable with WT. For NNK, the differences were the most striking as, although variant S298R showed a similar mutagenic activity compared with WT, all other variants had a reduced capacity to bioactivate this mutagen. Remarkably, in the case of T83M and C406Y, no mutagenicity could be detected.

I386F and R456H, two variants with perturbed expression of holoenzyme, showed no mutagenic activity for any of the three pre-mutagens, except for I386F with 2AA being significantly lower than WT.

2.3.3. Multivariate analysis of data

To compare all obtained experimental data, a multivariate analysis was performed. The polymorphic CYP1A2 variants were numbered from 2 to 7, whereas variants I386F and R456H were not included in the analysis. The different parameters, such as V_{max}, K_M, relative reaction velocities, IC_{50} and mutagenic activities, were designated A to P (Table 1).

2.3.3.1. Principal component analysis (PCA)

Principal component analysis (PCA), a procedure that transforms a high number of possibly correlated variables into a reduced number of uncorrelated variables denominated by principal components, was used to project present date from a 16-dimensional (activity parameters) space into a 2-dimensional space for ease of visualization (Figure 2). After a two-variance normalization, the first analysis was by a Scree plot analysis (not shown). This analysis indicated that the first principal component retains 94.5 % of the initial variance; therefore this single principal component explains almost all significant variations in the data. The PCA analysis itself seems to indicate that

the trend of variation that governs our data set stems from the difference between all WT-like enzymes [WT, no. 3 (S212C), no. 4 (S298R), no. 6 (I314V) and no. 7 (C406Y)], and non WT-like enzymes [variant no. 5 (G299S) and variant no. 2 (T83M)], as shown in Figure 2.

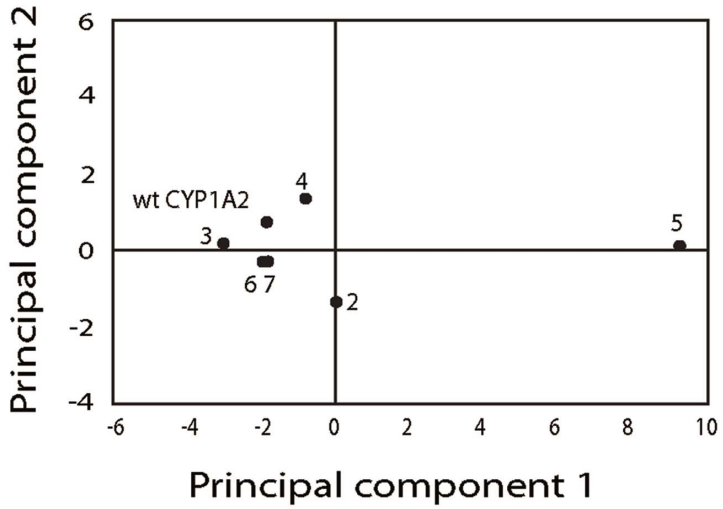

Figure 2. Principal component analysis (PCA) plot of the CYP1A2 variants activity normalized data set for the first two principal components (first principal component retains 94.5 % of the initial variance).

2.3.3.2. Multidimensional scaling (MDS)

For our data set, two different multidimensional scaling (MDS) configuration plots were constructed. In the first MDS configuration plot, CYP1A2 variants were compared using all activity parameters (Figure 3a). This plot shows how the enzymes were similar/dissimilar for all assayed activities. Kruskal's stress value was 0.002. The dendrogram depicted in Figure 3b deduced from MDS distances, showed that variants no. 2 (T83M) and no. 4 (S298R) were very similar and both were found slightly different from WT and variants no. 3, no. 6 and no. 7 (S212C, I314V, C406Y). Variant no. 5 (G299S) appears, again like in the PCA analysis, to constitute itself a particular enzyme, and different from all the others.

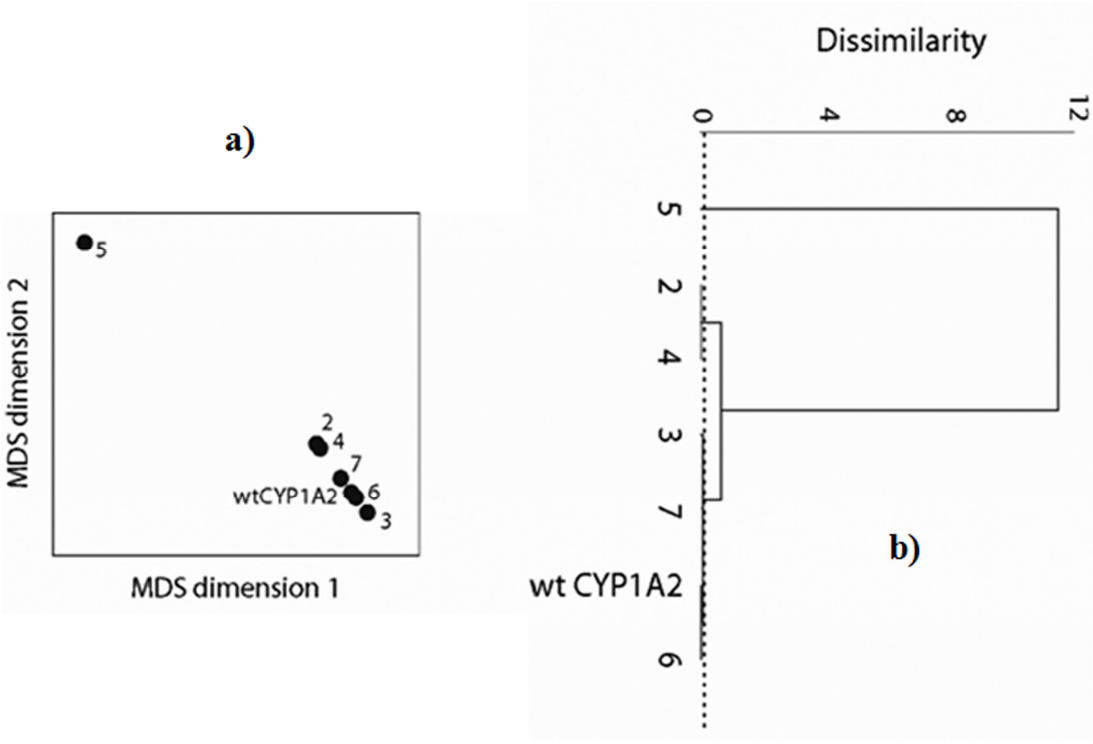

Figure 3. (**a**) Multidimensional scaling (MDS) configuration plot of the CYP1A2 variants activity normalized data set for the different activities A to P. (**b**) Dendrogram obtained from the distances between all CYP1A2 enzymes assayed in the MDS configuration plot.

The second MDS configuration plot depicted in Figure 4 compares the different activity parameters for the assayed CYP1A2 enzymes. This plot shows the force of activities in scrutinizing between the different CYP1A2 enzymes. Kruskal's stress value was 0.207, slightly over the typical value of 0.15; however,we are confident that this plot is meaningful. Three clusters could be deduced from the MDS configuration plot, which shows the distances between the activities for all assayed enzymes. Two objects were located in very separate and opposite areas of the graph, namely parameter C (EROD V_{max}) and parameter M (NNK mutagenic activity). All other parameters aggregated together in the central area of the two-dimensional plot.

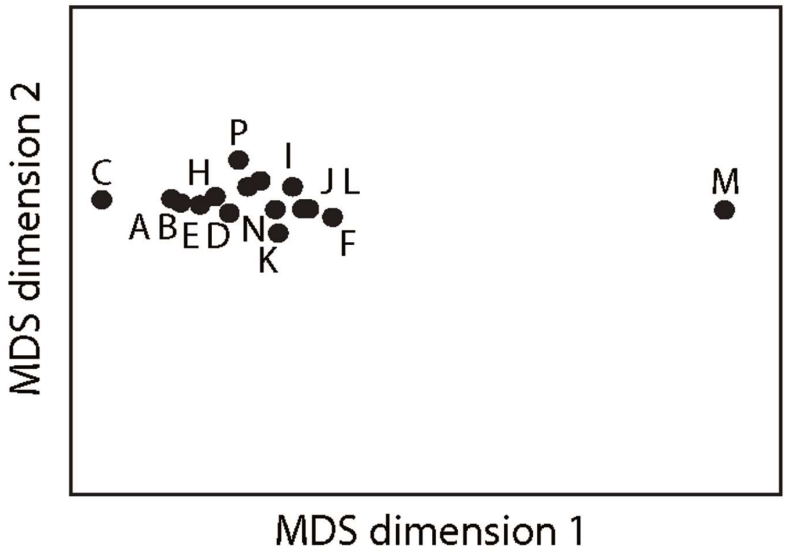

Figure 4. Multidimensional scaling (MDS) configuration plot of activities A to P for the different CYP1A2 variants.

Table I. Data set of all obtained activity parameters.

		MROD V_{max} pmol R per min		MROD K_M µM		EROD V_{max} pmol R per min		EROD K_M µM		CECOD V_{max} µM CHC per min		CECOD K_M µM		Phenacetin 20 µM pmol paracetamol per min		Phenacetin 200 µM pmol paracetamol per min	
		A		**B**		**C**		**D**		**E**		**F**		**G**		**H**	
		average	std desv	average	std desv	average	std desv	average	std desv	average	std desv	average	std desv	average	std desv	average	std desv
WT	**1**	2.55	0.10	0.59	0.06	0.75	0.04	1.16	0.16	3.37	0.04	5.00	0.23	31.47	3.45	84.28	5.87
T83M	**2**	4.65	0.34	1.02	0.15	1.41	0.12	1.71	0.34	5.30	0.05	3.53	0.13	24.74	3.24	98.54	12.31
S212C	**3**	2.78	0.15	0.58	0.08	0.84	0.04	1.14	0.14	2.99	0.07	4.48	0.40	26.67	1.53	89.63	5.89
S298R	**4**	1.86	0.05	0.43	0.03	0.53	0.03	1.23	0.17	3.25	0.09	5.23	0.51	17.94	2.00	50.78	9.16
G299S	**5**	3.38	0.20	0.88	0.11	0.72	0.02	1.53	0.13	4.13	0.08	4.04	0.29	20.03	1.80	74.26	5.13
I314V	**6**	5.23	0.61	1.47	0.32	1.28	0.08	2.11	0.28	7.48	0.11	5.17	0.30	30.49	5.30	88.30	2.02
C406Y	**7**	3.63	0.22	0.92	0.12	0.76	0.03	1.68	0.15	4.14	0.08	4.95	0.39	14.95	0.75	69.57	1.73

		Clozapine 50 µM pmol N-demeth product per min		Clozapine 250 µM pmol N-demeth product per min		2AA rev per nmol 2AA		IQ rev per nmol IQ		NNK rev per µmol NNK		IC50 MROD µM	IC50 EROD µM	IC50 CEC µM
		I		**J**		**K**		**L**		**M**		**N**	**O**	**P**
		average	std desv	average	std desv	average	std desv	average	std desv	average	std desv			
WT	**1**	64.8	5.3	140.3	6.6	10902	328	318	26	1098	95	0.0025	0.2388	0.1416
T83M	**2**	41.4	2.2	117.1	5.9	8361	398	215	13	-	-	0.0025	0.2043	0.0979
S212C	**3**	48.3	0.2	124.1	4.1	12662	440	313	48	599	133	0.0024	0.1686	0.0599
S298R	**4**	37.5	1.0	152.0	7.2	8274	227	232	39	1151	85	0.0025	0.1603	0.0676
G299S	**5**	46.9	2.3	157.1	2.4	4657	447	244	16	575	65	0.0025	0.1577	0.0733
I314V	**6**	42.4	1.1	91.2	9.7	11793	153	216	29	694	94	0.0027	0.2811	0.1265
C406Y	**7**	28.6	1.4	112.0	2.8	9561	494	274	27	-	-	0.0024	0.1260	0.0813

Abbreviations: 2AA, 2-aminoanthracene; CEC, 3-cyano-7-ethoxycoumarin; CECOD, 3-cyano-7-ethoxycoumarin O-dealkylation; CHC, 3-cyano-7-hydroxycoumarin; EROD, 7-ethoxy-resorufin O-dealkylation; IQ, 2-Amino-3-methylimidazo(4,5-*f*)quinoline; MROD, 7-Methoxy- resorufin O-dealkylation; N-demeth, N-demethylation; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; R, resorufin; rev, revertant colonies; WT, wild type.

2.4. DISCUSSION

Polymorphisms in xenobiotic-metabolizing enzymes have been recognized as a main factor in inter-individual variability in drug and xenobiotic responses and susceptibilities. In particular, CYP1A2 is involved in the metabolism of a large number of drugs and chemical carcinogens. A recent study found significant differences in CYP1A2 activity between ethnic groups (Swedes and Koreans), not related to environmental factors [37]. CYP1A2 polymorphisms could also be related to the toxicity of leflunomide in patients with rheumatoid arthritis [38] and seem to have a role in the efficacy of treatment of patients with antipsychotics, such as clozapine and olanzepine [39]. Several studies indicated implications of CYP1A2 polymorphisms in cancer susceptibility, namely for pancreatic cancer [40] and bladder cancer [41].

To date, 39 single-nucleotide polymorphisms of human CYP1A2 have been reported (from which 36 haplotypes were determined), describing alterations on DNA sequence levels and in some cases their epidemiological frequencies. From the 17 known non-synonymous protein polymorphic alleles, 5 have not been characterized regarding their enzymatic activity (S212C, F21L, S298R, I314V and R431W; see Table 2). Two of these were included in this study. Formerly, other non-synonymous protein forms were probed with only two substrates, except for I386, D348W and C406, which were analyzed with more substrates (see Table 2). In this report, we describe the thorough characterization of 8 polymorphic variants of human CYP1A2, with 16 different activity parameters.

Table 2. CYP1A2 non-synonymous protein polymorphic alleles: comparison between current and former studies.

Non synonymous protein polymorphism	Other studies		This study
	Substrates	**References**	**Substrates**
S18C	_____	Solus, *et al* [42]	_____
F21L	_____	Huang, *et al* [43]	_____
P42R	ER and Phenacetin	Saito, *et al* [23]	_____
T83M	ER and Phenacetin	Murayama, *et al* [22]	8 [a]
E168Q	ER and Phenacetin	Murayama, *et al* [22]	_____
F186L	ER and Phenacetin	Murayama, *et al* [22]	_____
S212C	ER and Phenacetin	Murayama, *et al* [22]	8 [a]
S298R	_____	Solus, *et al* [42]	8 [a]
G299S	ER and Phenacetin	Murayama, *et al* [22]	8 [a]
I314V	_____	Solus, *et al* [42]	8 [a]
D348N	IQ, MeIQ, MeIQx, PhIP, Glu-1-P and Phenacetin	Zhou, *et al* [7]	_____
R377Q	ER and Phenacetin	Saito, *et al* [23]	_____
I386F	IQ, MeIQ, MeIQx, PhIP, Glu-1-P and Phenacetin	Zhou, *et al* [7]	8 [a]
C406Y	IQ, MeIQ, MeIQx, PhIP, Glu-1-P and Phenacetin	Zhou, *et al* [7]	8 [a]
R431W	_____	Zhou, *et al* [7] Chevalier, *et al* [44]	_____
T438I	ER and Phenacetin	Murayama, *et al* [22]	_____
R456H	ER and Phenacetin	Saito, *et al* [23]	8[a]

Abbreviations: ER, ethoxyresorufin; IQ, 2-amino-3-methylimidazo(4,5-f)quinoline. Glu-1-P, 2-amino-6-methyldlpyrido[1,2-a:30,20-d]imidazole; MelQ, 2-amino-2,4-dimethylimidazo[4,5-f]quinoline; MelQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; PhlP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

[a] This study, see Table 1 and Figure 1.

The CYP content was similar to WT for all variants, except for I386F and R456H. These variants showed severely reduced or absent holoenzyme, respectively, in bacterial cells, and both were absent in membrane fractions, indicating highly unstable holoenzymes. The CYPOR expression levels in membrane preparations when co-

expressed with the various CYP1A2 variants were similar to the WT strain. The CYPOR/CYP stoichiometry is recognized as a critical factor for the catalytic properties of CYP enzymatic system [26]. CYPOR/CYP ratios for the different variants were found to be in the range of those observed in human liver microsomes. These results first indicated that coexpression of these CYP1A2 variants with CYPOR represented an adequate approximation of the *in vivo* situation, and second that in principle the observed alterations in CYP1A2 activity could be solely ascribed to the CYP1A2 variant.

CYP1A2 variants were subjected to several activity assays using eight substantially different substrates, in an attempt to discriminate between allelic variants as much as possible. Different substrate classes with different types of CYP1A2-mediated reactions were used, namely: *O*-dealkylation (MR, ER, CEC and phenacetin); *N*-demethylation (clozapine); and *N*-hydroxylation (NNK, IQ and 2AA).

As a result, a very heterogeneous activity data set could be generated for CYP1A2 allelic forms. To thoroughly analyse this diverse data set, multivariate statistical analysis, a very useful statistical tool, was performed on this data set. Variants I386F and R456H were excluded from this analysis because of the lack of detectable holoenzyme. Differences between polymorphic CYP1A2 variants and WT were analyzed globally by principal component analysis and multidimensional scaling, two statistical tools of complementary interest as was shown in several fields [45, 46], including the recent exploration of protein structure space [47, 48].

All applied statistical analyses indicated that variant S212C (no. 3) represents the most similar enzyme to WT. Murayama *et al* [22] found also similar relative activities to WT for this variant, using two substrates, namely ER and phenacetin. Both PCA and MDS analysis indicate that variants I314V (no. 6) and C406Y (no. 7) were similar to WT. In a previous report [7], no major differences were observed for variant C406Y activity,

corroborating our results. It is interesting to note that the lack of bioactivation of NNK of this variant described in this report coincides with observations from previously referred authors. This group described altered catalytic specificity of C406Y for three heterocyclic amine pre-mutagens, other than NNK. They proposed that possible changes in the regioselectivity and regiospecificity of the reactions might have lead to an altered metabolite profile. This theory seems to be reinforced by our observation of extra metabolites in phenacetin *O*-dealkylation for this variant. Variant S298R (no. 4) appears to be a slightly different enzyme from WT, both from MDS and PCA analysis. This variant is located on the surface of the heme domain, on the proximal side of the heme (see Figure 5). On the basis of alignment and superposition of the human CYP1A2 crystal structure [24], with the structure of the CYP BM3 heme and FMN-binding domains [49], S298 seems also to be located near the CYPOR interaction area. The S298R amino-acid change might influence the interaction of CYP1A2 with its redox partner, explaining the observed differences. Both MDS and PCA analysis indicated that variant T83M (no. 2) is a significantly different enzyme from WT. On the basis of the human CYP1A2 structure published by Sansen *et al* [24], and the description of substrate entrance and product exit channels of CYPs by Cojocaru *et al* [50], it seems likely that this residue is located in a substrate entrance/product exit channel. Finally, G299S (no. 5) is definitely indicated as the most distinguishable variant, of all studied variants in thisstudy. This is consistent with all performed statistical analyses, and it seems to indicate not only a significant different behaviour from WT but also from all other analyzed variants. G299 is located beside S298, with the possibility of G299S disturbing the interaction of CYP1A2 with CYPOR (*vide supra*). From the MDS plot for substrates (Figure 4), it is interesting to note that NNK (mutagenic activity) represents the most discriminative

activity parameter among the CYP1A2 variants of a heterogeneous set of 16 activity parameters.

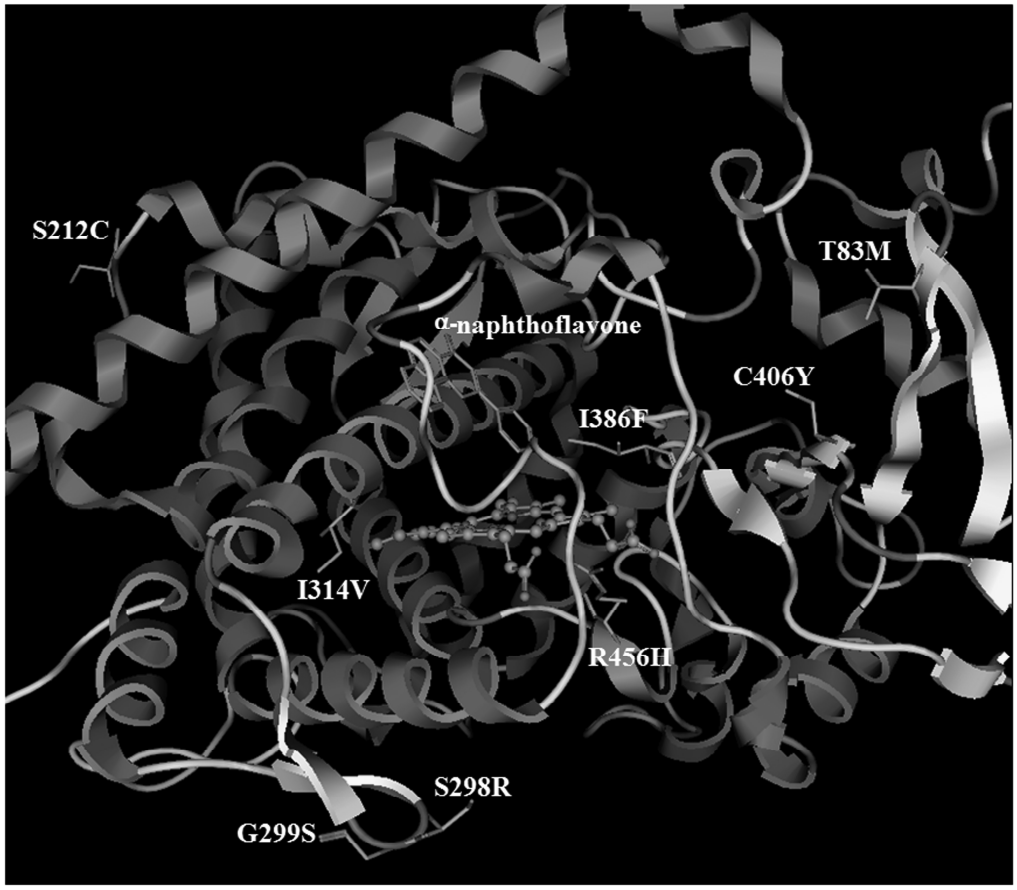

Figure 5. Localization of altered amino acids of the selected CYP1A2 polymorphic variants using the human CYP1A2 crystal structure published by Sansen, *et al* [24]. (This figure was generated using PDB file no. 2H14).

Variants I386F and R456H showed to be two particular cases, both showing severe problems with holoprotein stability. In a previous study by Zhou *et al* [7], variant I386F showed a relative low expression level of holoenzyme when expressed in bacterial cells. The I386 residue is located in the substrate recognition site 5 and based on the CYP1A2 crystal structure published by Sansen *et al* [24], very close to the catalytic center, only 3.8 Å apart from the cocrystallized substrate α-NF and around 4 Å from the heme. In mammalian CYP structures, known up to now, the common structural features

of the heme surroundings is formed by the I-helix, the substrate recognition site 5 and the BC loop [51]. The I386F substitution represents the replacement of an aliphatic side chain with an aromatic side chain, which could distort the heme-anchoring structure, explaining the observed highly unstable I386F holoprotein. This is supported by the increase in the 420- nm peak in the CO-difference spectrum of this variant, indicating dislocated heme positioning. In the case of R456H, there was no detectable holoenzyme, both in whole cells and membrane fractions, with a concomitant increase in the 420-nm peak in the CO-difference spectrum, observations in accordance with previous study by Saito *et al* [23]. Previous studies with the rat orthologous of CYP1A2, suggested that the region containing this amino acid is crucial in heme binding and its interaction with CYPOR [52, 53]. The substitution of R456 in humans (corresponding to rat R454) showed markedly reduced activities. In addition, a positively charged residue, the position of which is variable in the C-terminal end of the substrate recognition site 5 region, has been identified by protein sequence alignments and is conserved in 97.7% of a total of 6379 CYP sequences [51]. The location of this residue in all-known crystal CYP structures, including the CYP1A2 structure indicates that this residue interacts with the 7′-propionate of the heme. A previous reported work, which compared four CYP crystal structures and 200 sequences, suggested a functional conserved heme interacting arginine that is also involved in the elimination of water from the active site [54].

In summary, we successfully expressed eight allelic variants of CYP1A2 with human CYPOR and could measure multiple activities parameters of eight structural different substrates. Two of these variants, namely I386F and R456H, were enzymatically inactive. The collected heterogeneous data set from the remaining variants was analyzed using several multivariate analysis tools. This analysis indicated that variant G299S (no. 5) seems to have substantial altered catalytic properties in comparison with WT, whereas

variant T83M (no. 2) is slightly but significantly different from the WT. Moreover, an important residue of the scaffold for proper heme binding and stabilization, R456, could be identified in CYP1A2.

At present, it is acknowledged that polymorphisms in CYP1A2 do not profoundly penetrate into the phenotype level [55]. However, we have shown in this study that although the eight variants cause, seemingly on the whole, relative minor activity changes (besides I386F and R456H), some variants can have profound effects on particular CYP1A2 mediated biotransformation, as was demonstrated for the bioactivation of NNK. As such, the results indicated that several of the studied allelic forms can have pharmokinetic and/or toxicokinetic consequences. Moreover, our results seem to indicate and/or confirm structural characteristics and features of the CYP1A2 enzyme. As such, studying polymorphic CYP activity is also a driving tool for improving our knowledge on the molecular mechanism of the functioning of the human CYP1A2 and the CYP enzyme complex in general.

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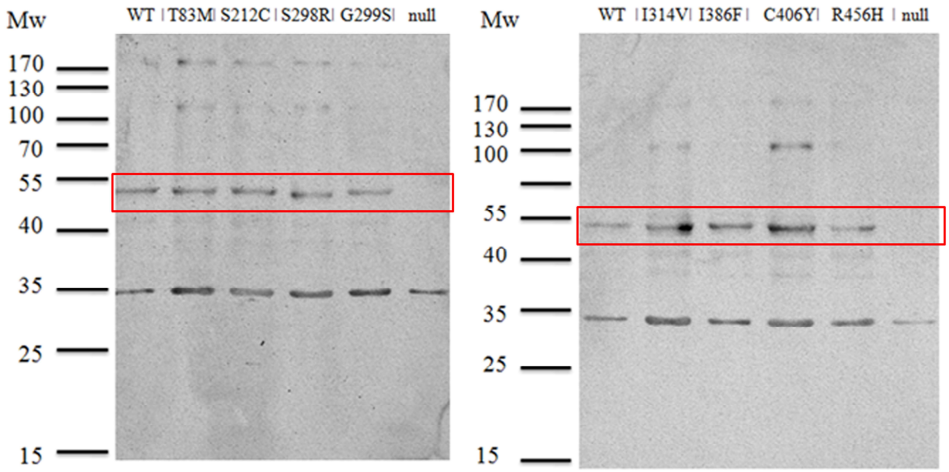
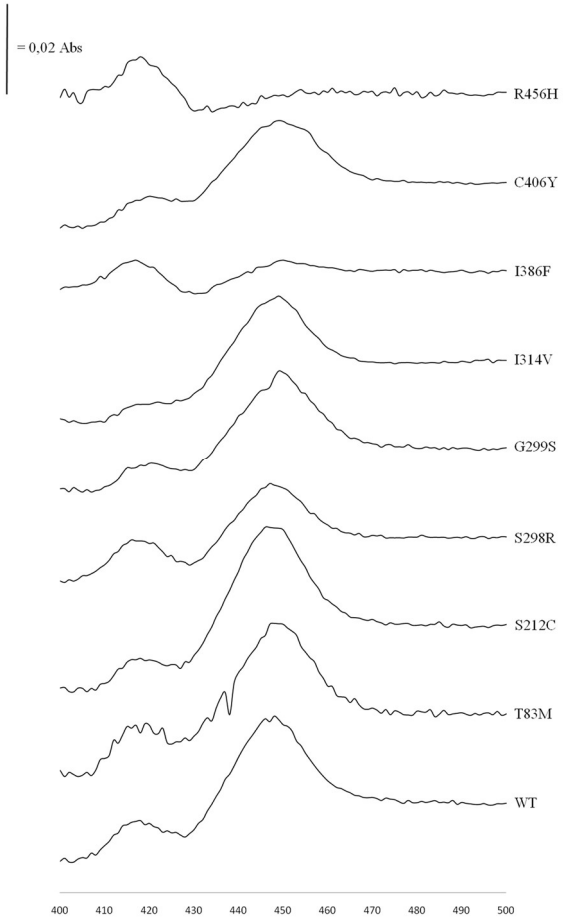
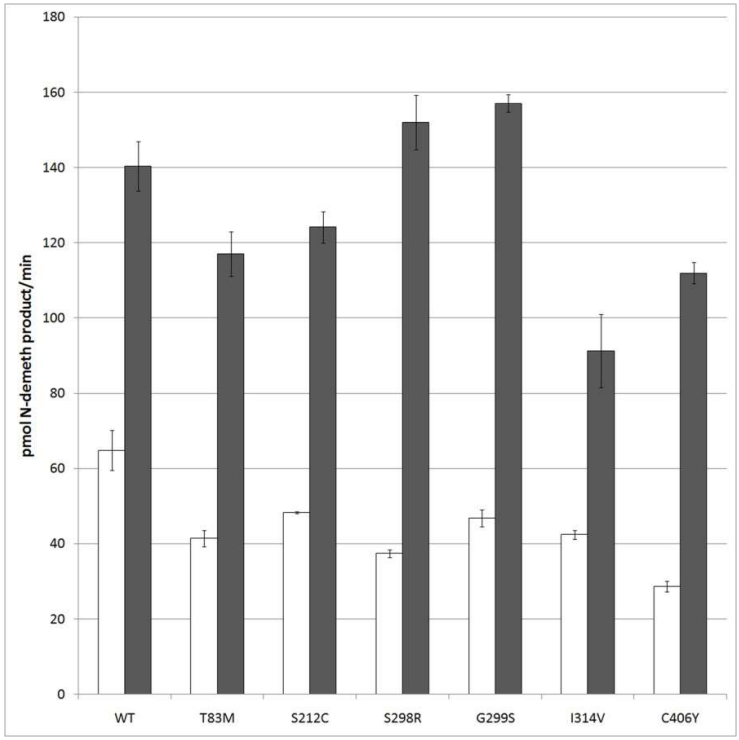
2.5. SUPPLEMENTARY INFORMATION



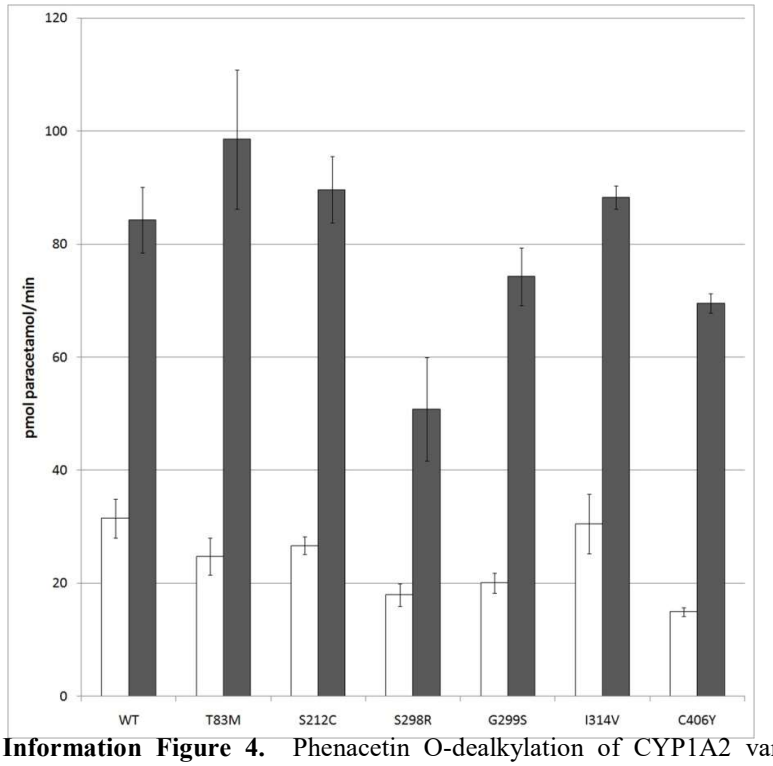
Supplementary Information Figure 1. Immunoblot analysis of CYP1A2 polymorphic variants and WT expressed in *E. coli*(Each lane contained 10 µg of membrane fraction; Mw: Molecular weight marker,values in kDa. Boxed signals indicating position of CYP1A2).



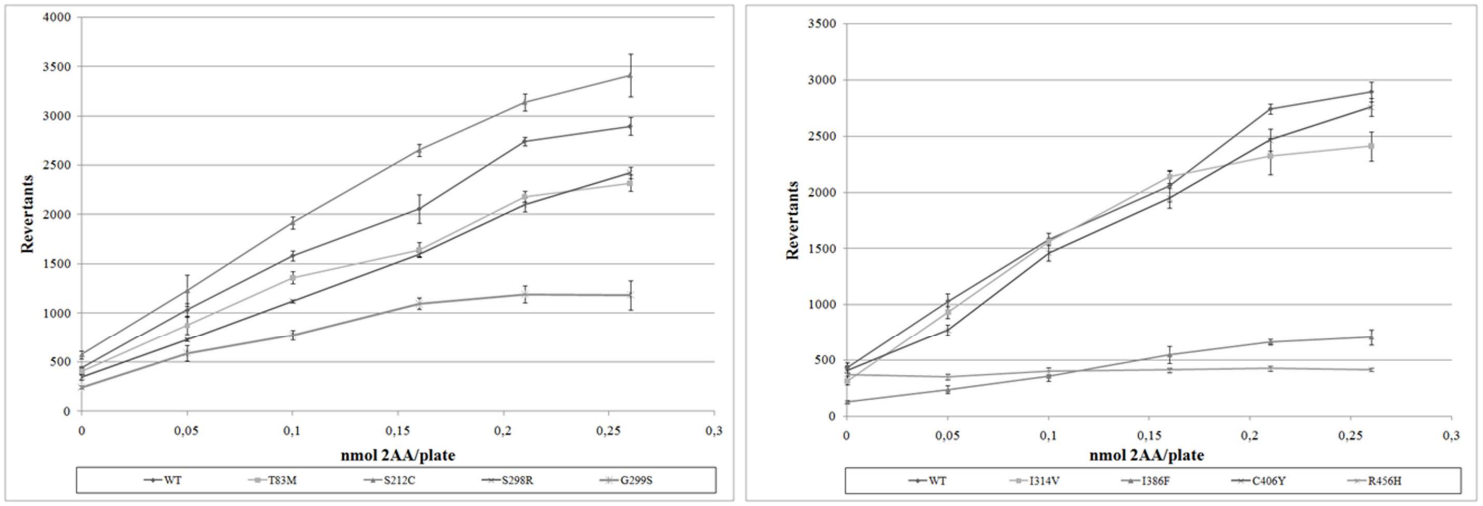
Supplementary Information Figure 2. CO-difference spectra of *E. coli* BTC whole cells expressing the different variants of CYP1A2. (Spectral data was normalized for cell density; spectra represents n=3. The standard deviation of [CYP] did not exceeded 2,5 % of mean)



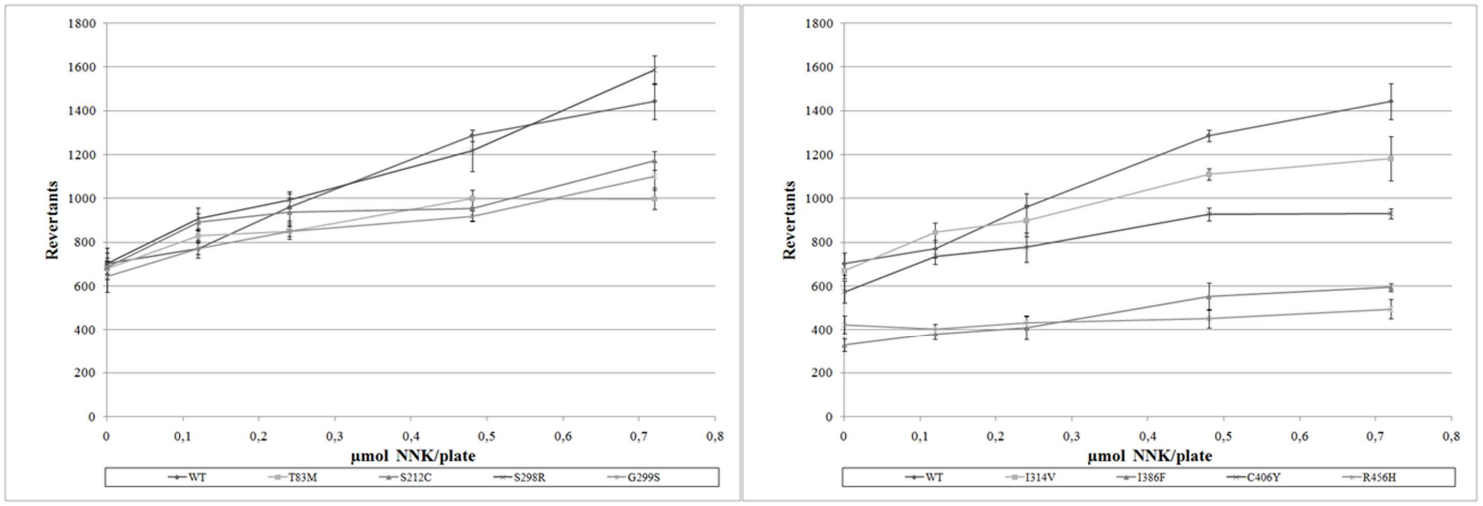
Supplementary Information Figure 3. Clozapine N-demethylation of CYP1A2 variants. (Relative activities determined with 50 µM [white bars] and 250 µM [grey bars] of clozapine; Error bars represent standard deviations.)



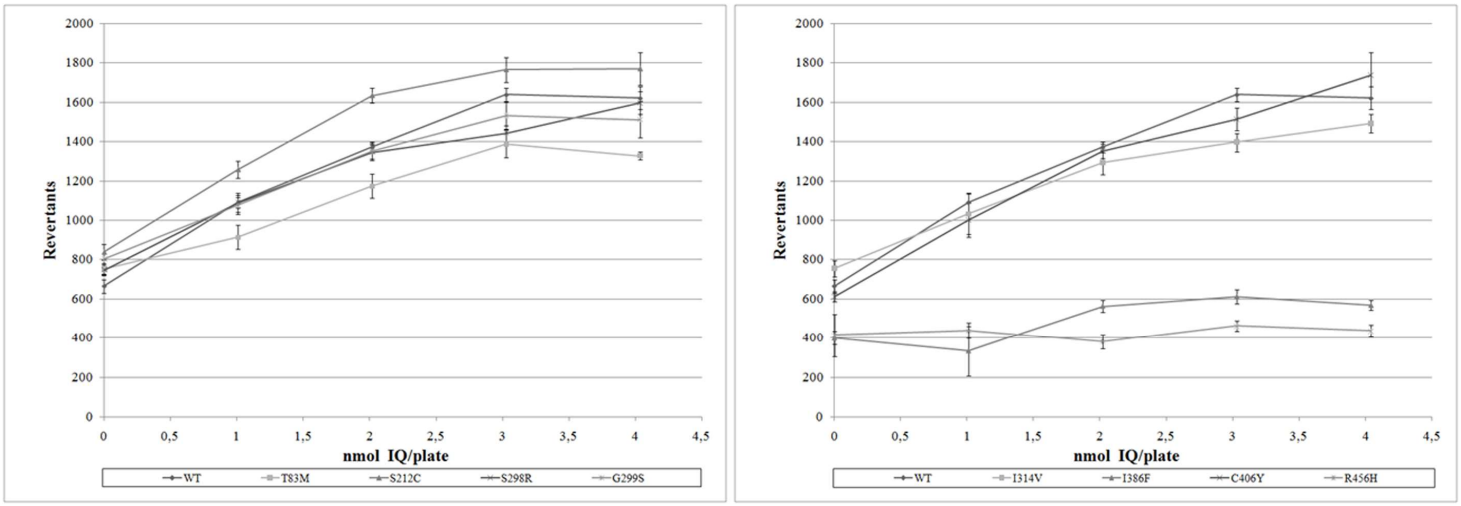
Supplementary Information Figure 4. Phenacetin O-dealkylation of CYP1A2 variants. (Relative activities determined with 20 µM [white bars] and 200 µM [grey bars] of phenacetin. Error bars represent standard deviations.)



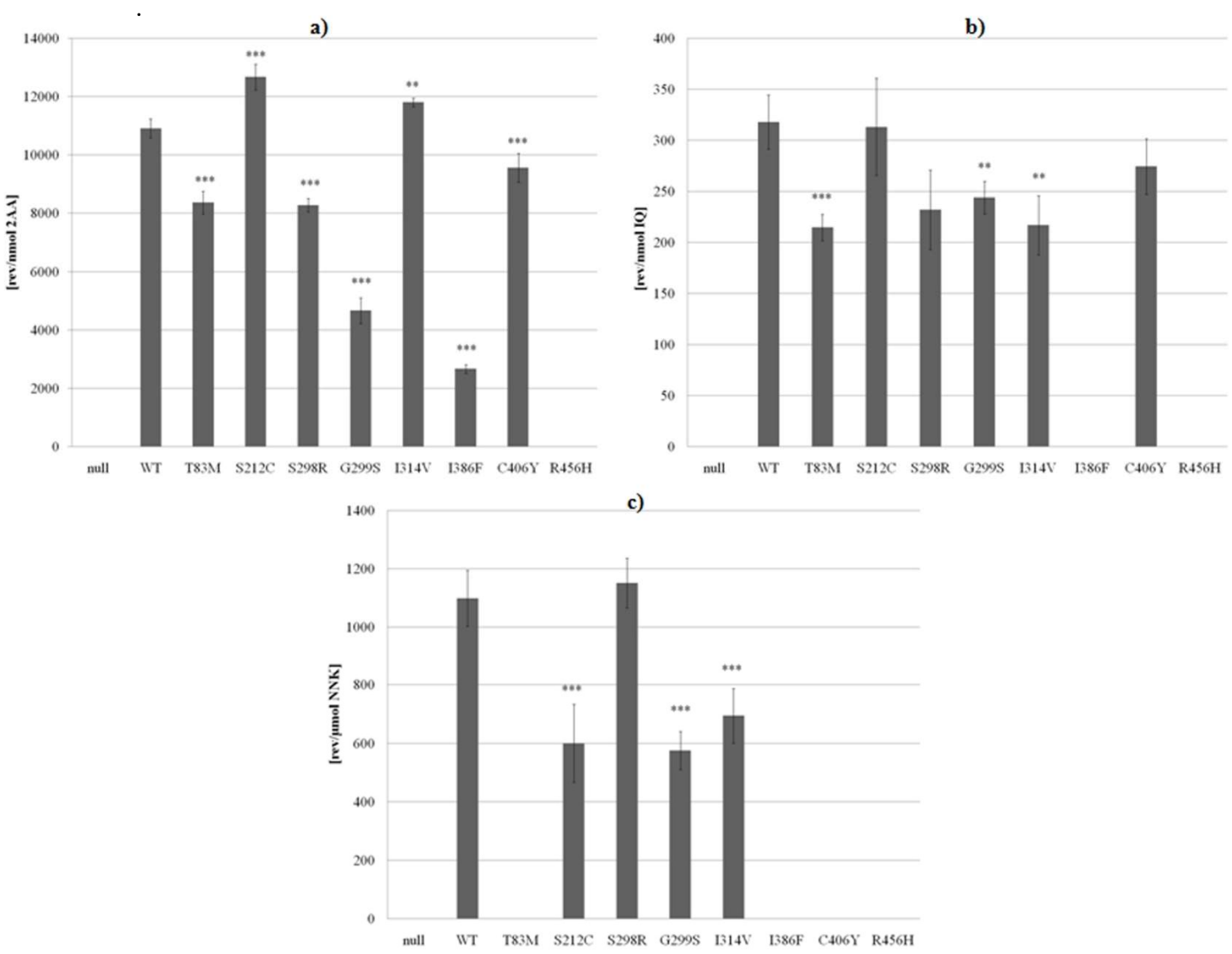
Supplementary Information Figure 5. Bioactivation capacity of CYP1A2 polymorphic variants. Dose-response curves for 2AA.



Supplementary Information Figure 6. Bioactivation capacity of CYP1A2 polymorphic variants. Dose-response curves for IQ.



Supplementary Information Figure 7. Bioactivation capacity of CYP1A2 polymorphic variants. Dose-response curves for NNK.



Supplementary Information Figure 8. Bioactivation capacity of CYP1A2 polymorphic variants of the premutagens a): 2AA; b): NKK and c): IQ (expressed in number of revertant colonies per nmol or µmol of compound; n=3, ** p< 0.01 and *** p<0.001) (). Error bars represent standard deviations.

Supplementary Information Table I. Reference SNP IDs, Allele frequencies and Mutagenesis Primers used for site directed mutagenesis.

Allele	Reference SNP ID	Allele frequency	Primers for site-directed mutagenesis (mutated nucleotide is designated underlined)		Amino acid substitution
CYP1A2*9	[1]	[1]	Forward	5′ GATCCGCATTGGCTCCA**T**GCCCGTGCTGGTGCTG 3′	T83M
			Reverse	3′ CTAGGCGTAACCGAGGT**A**CGGGCACGACCACGAC 5′	
CYP1A2*12	[1]	[1]	Forward	5′ CAGCACTTCCCTGAG**T**GTAGCGATGAGATGCTC 3′	S212C
			Reverse	3′ GTCGTGAAGGGACTC**A**CATCGCTACTCTACGAG 5′	
No designation	rs17861157	3 % [2]	Forward	5′ GAAGGGGCCTAGAGCC**C**GCGGCAACCTCATCCCAC 3′	S298R
			Reverse	3′ CTTCCCCGGATCTCGG**G**CGCCGTTGGAGTAGGGTG 5′	
CYP1A2*13	rs35796837	1% [2]	Forward	5′ GGGCCTAGAGCCAGC**A**GCAACCTCATCCCACAG 3′	G299S
			Reverse	3′ CCCGGATCTCGGTCG**T**CGTTGGAGTAGGGTGTC 5′	
No designation	rs28399418	[1]	Forward	5′ GTCAACCTTGTCAATGAC**G**TCTTTGGAGCAGGATTTGAC 3′	I314V
			Reverse	3′ CAGTTGGAACAGTTACTG**C**AGAAACCTCGTCCTAAACTG 5′	
CYP1A2*4	[1]	[1]	Forward	5′ CCTTCTTGCCCTTCACC**T**TCCCCCACAGCACAACAAG 3′	I386F
			Reverse	3′ GGAAGAACGGGAAGTGG**A**AGGGGGTGTCGTGTTGTTC 5′	
CYP1A2*5	rs55889066	[1]	Forward	5′ CTACATCCCCAAGAAATGCT**A**TGTCTTCGTAAACCAGTGG 3′	C406Y
			Reverse	3′ GATGTAGGGGTTCTTTACGA**T**ACAGAAGCATTTGGTCACC 5′	
CYP1A2*8	[1]	[1]	Forward	5′ GGCATGGGCAAGC**A**CCGGTGTATCGGG 3′	R456H
			Reverse	3′ CCGTACCCGTTCG**T**GGCCACATAGCCC 5′	

([1] not yet designated (SNP ID) or determined (allele frequency))

([2]allele frequencies obtained from: *http://www.genecards.org/index.shtml*)

CHAPTER 3

Functional characterization of eight human cytochrome P450 1A2 variants: The role of cytochrome b_5

Adapted from: Palma, B.B.; Silva e Sousa, M.; Urban, P.; Rueff, J.; Kranendonk, M.; *"Functional characterization of eight human CYP1A2 variants: the role of cytochrome b_5"* Pharmacogenet Genomics. 2013 Feb; 23(2):41-52.

ABSTRACT

Background Interindividual variability in cytochrome P450 (CYP)-mediated xenobiotic metabolism is extensive. CYP metabolism requires two electrons, which can be donated by NADPH cytochrome P450 oxidoreductase (CYPOR) and/or cytochrome b_5 (b_5). Although substantial number of studies have been reported on the function and effect of b_5 in CYP-mediated catalysis, its mode of action is still not fully understood.

Objective The aim of this work was to examine the effect of b_5 on the activities of eight natural-occurring variants of human CYP1A2, namely: T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H.

Materials and Methods An approach, as used in our former study was applied, coexpressing these polymorphic CYP1A2 variants separately with CYPOR and b_5 in the bacterial cell model BTC-CYP. For each variant, 16 different activity parameters were measured, using eight different substrates. This heterogeneous data set was merged with the one of our former study (i.e. without b_5) and a multivariate analysis was carried out.

Results This analysis indicated that b_5 seems to have the ability to affect CYP1A2 variants to behave more like the wild-type variant. This was especially the case for variant I386F, for which the presence of b_5 was crucial to show activity. Variants T83M and C406Y showed considerably different activity-profiles when in the presence of b_5. Furthermore, our data seem to implicate CYP1A2 residue G299 in its interaction with CYPOR and/or b_5.

Conclusion Results indicate the ability of b_5 to affect CYP1A2 variants to behave more like the wild-type variant, attenuating detrimental effects of structural mutations of these variants, seemingly through extensive allosteric effects.

3.1 INTRODUCTION

Cytochrome P450 (CYP) is a large superfamily of enzymes that are responsible for the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds [1]. In this enzymatic system, CYPs from families 1-3 mediate 70-80% of the phase I-dependent metabolism of clinical drugs [2] participating in the metabolism of a large number of structurally different xenobiotics. All genes that encode CYPs from families 1-3 are polymorphic [3]. Human CYP polymorphisms include not only mutations in noncoding regions (promoter, intron) but also in the protein-coding sequences. Mutations in noncoding sequences may lead to altered expression levels, whereas mutations in protein-coding sequences may change the structure and catalytic properties of the enzyme [4].Currently, it is generally accepted that genetic variations of genes encoding drug-metabolizing enzymes play a major role in the outcome of drug treatment. CYP polymorphism has been indicated as a major reason why substantial numbers of patients do not respond, respond partially, or present adverse drug reactions [5, 6]. Therefore, it is important to understand the effects of CYP polymorphism to improve our comprehension of interindividual differences in the outcome of drug treatments, as well as in chemical exposure in general [7].

Human CYP1A2 is almost exclusively expressed in the liver, representing ~15% of its total CYP content [8]. CYP1A2 catalyze the bioactivation of ~17% of human precarcinogens [9], such as aromatic and heterocyclic amines [10], and mediates the metabolism of about 15% of clinical drugs [11], such as clozapine [12], lidocaine [13], phenacetin [14], propranolol [15] and tacrine [16]. Differences between individuals in CYP1A2 activity are well known: up to 60-fold variations have been reported [8, 17]. To date, 36 CYP1A2 haplotypes have been catalogued by the Human Cytochrome P450 Allele Nomenclature Committee (*http://www.cypalleles.ki.se/cyp1a2.htm*). Recently, our

group reported on a study on the functional characterization of eight CYP1A2 polymorphic variants, relative to CYP1A2 wild-type (1A2*1) (WT) form, using a structurally diverse set of substrates, measuring 16 different activity parameters [18]. Our analysis indicated variants G299S [CYP1A2*13] and T83M [CYP1A2*9] to be significantly different from the WT variant, and could be interpreted structurally, on the basis of their location in the CYP1A2 protein. In addition, R456 could be identified as an important residue for proper heme binding and stabilization in the CYP1A2 protein [18].

Microsomal CYP metabolism requires a coupled supply of electrons, which are donated by the auxiliary protein NADPH cytochrome P450 oxidoreductase (CYPOR) [19]. Similar to CYP, CYPOR is a membrane-bound protein. The reductase contains two flavin moieties, FMN and FAD, essential for the sequential one-electron transfer to CYP, originating from NADPH [20]. Another heme protein has been shown to be important for CYP-mediated reactions, namely, cytochrome b_5 (b_5). The discussion of b_5 involvement in CYP reactions has been controversial since it was first suggested by Hildebrandt and Estabrook [21]. The heme protein b_5 is a ubiquitous small protein of ~17 kDa that is present throughout the phyla. In all species and tissues, b_5 is anchored in the membrane by a hydrophobic carboxyl-terminal end, except for erythrocytes, in which a soluble form is found [22]. Both these forms of b_5 carry a prosthetic heme group and can exist in oxidized and reduced states [23]. Physiologically, b_5 can be reduced both by cytochrome b_5 reductase (NADH dependent) and by CYPOR [24]. Besides its involvement in CYP-mediated biotransformation, b_5 is also involved in lipid biosynthesis, serving as the electron donor to at least three separate microsomal desaturases involved in the fatty acid metabolism [25], and is known to be involved as an electron transfer partner in a large number of other oxidative (non-CYP) reactions in biological tissues [26].

Several studies using different types of in-vitro systemshave reported that b_5 could modulate specific activities of several CYPs. The role of b5 in modulating CYP17A1 activity (17α-hydroxylation and 17,20-lyase activity of C_{21}-steroids) is now fairly established [27]. Other studies have reported the effects of b_5 on the catalytic properties of xenobiotic metabolizing CYPs such as CYP3A5 [28], CYP3A4 [28-31], CYP2C9 [29, 31], CYP2B4 [32], CYP2A6 [29, 33] and CYP2E1 [29, 33-35]. Depending on the xenobiotic CYP involved, substrate, and experimental (in-vitro) conditions, b_5 has been shown to stimulate, inhibit or not to interfere with CYP reactions [36, 37]. Beside this CYP-dependent and/or substrate-dependent effect through mechanisms that are still not well understood, b_5 might have differentiated effects on genetic variants of the same CYP isoforms, a factor that is important in the understanding of the variability in drug therapy and chemical exposure in general.

Therefore, the primary aim of the present study was to examine the effect of b_5 on the activities of eight natural occurring variants of human CYP1A2 to determine the role of b_5 in the variability of CYP1A2-mediated biotransformation. For this purpose, we used the established *Escherichia coli* BTC cell model [38], which has been shown to functionally and correctly coexpress human CYP together with CYPOR and b_5 in stoichiometries which reflect those found in the human liver [33]. CYP1A2-WT and its genetic variants T83M [CYP1A2*9], S212C [CYP1A2*12], S298R [no allele designation], G299S [CYP1A2*13], I314V [no allele designation], I386F [CYP1A2*4], C406Y [CYP1A2*5] and R456H [CYP1A2*8] were coexpressed separately, with CYPOR and b_5 in this bacterial system. The same 16 different activity parameters were measured using the structural diverse set of eight substrates as in our former study [18]. This CYP1A2/b_5 activity data set was combined with the one of our former study (activity data obtained in the absence of b_5) [18], and subsequently examined together by

multivariate analysis. The result of this analysis were then interpreted using the CYP1A2 crystal structure [39].

3.2. MATERIALS AND METHODS

3.2.1. Reagents

L-Arginine, ampicillin, kanamycin sulfate, chloramphenicol, cytochrome *c*, isopropyl β-D-thiogalactoside (dioxane-free) (IPTG), thiamine, 2-aminoanthracene (2AA), glucose-6-phosphate, NADPH, ethoxyresorufin (ER), methoxyresorufin (MR), resorufin, phenylmethanesulfonyl fluoride (PMSF) and α-naphthoflavone (α-NF) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2-Amino-3-methylimidazo(4,5-*f*)quinoline (IQ) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). 3-Cyano-7-ethoxycoumarin (CEC) and 3-cyano-7-hydroxycoumarin (CHC) were obtained from BD Biosciences (Bedford, MA, USA). Phenacetin was obtained from Brocades-ACF (Maarssen, The Netherlands) and clozapine from Duchefa Farma bv. (Haarlem, The Netherlands). Bacto agar, bacto peptone, bacto tryptone and bacto yeast extract were obtained from Difco (Detroit, MI, USA). All other chemicals were of the highest quality.

3.2.2. Construction of CYPOR/b_5 expression plasmid pLCM_hB5_POR

The expression of the different CYP1A2 variants with CYPOR and human b_5 was achieved through the biplasmid coexpression system [33, 38], using the human CYP1A2 expression vectors of all variants of our former study [18] and a newly constructed plasmid, pLCM_hb5_PORwt, encoding human b_5 and CYPOR (see Figure, Supplemental digital content 1, *http://links.lww.com/FPC/A539*). The latter plasmid was

obtained through the PCR amplification of the pTAC_hB5 sequence of pLCM-b5-RED [33] using forward and reverse primers TACGTCTAGACACCAATGCTTCTGGCGTCA and TCAGGGATCCACTTCTGAGGAGGTGTTCAG. The PCR product was digested with BamHI and XbaI and subcloned in pUC18, and cDNA was verified by sequencing. Subsequently, pUC18_pTAC_hB5 was digested with BamHI and XbaI and the XbaI-BamHI fragment was isolated and ligated in pLCM_PORwt [40] previously opened by BamHI and XbaI digestion. The total pTAC_hB5_pTAC_PORwt DNA stretch of pLCM_hb_5_PORwt was verified by sequencing.

3.2.3. Bacterial coexpression of CYP1A2 variants with CYPOR and membrane preparation

The pCWh1A2 expression vectors encoding each of the different nine CYP1A2 variants [18] were combined separately with the plasmid pLCM_hb5_PORwt in BTC by electroporation. The control strain BTCb5Por was obtained by transfection with pLCM_hb5_PORwt and the empty pCW expression vector. Each strain was cultured in TB medium supplemented with peptone (2 g/l), thiamine (1 μg/ml), ampicillin (50 μg/ml), kanamycin (15 μg/mL), chloramphenicol (10 μg/ml), trace elements solution [41] (4 μl/ml), and 0.2 mmol/l isopropyl β-D-thiogalactoside (final concentrations). Cultures were started with 250 μl of –80 ºC glycerol stocks and cells were grown for 16 h at 28 ºC with moderate agitation.

All membrane preparations were obtained as described previously [18]. Briefly, cultures were harvested at 2772*g*, 20 min at 4 °C. The pellet was resuspended in TS buffer (75 mmol/l Tris-HCl, 250 mmol/l sucrose, pH 7.8). Lysozyme was added to a final concentration of 0.5 mg/ml and cells were incubated on a roller bench for 30 min at 4 °C. Subsequently, EDTA and protease inhibitor PMSF were added to a final concentration of

0.5 mmol/l each. Cell lyses was performed by freezing (–80 ºC) and thawing (1 cycle) and subsequent several short rounds (60 s) of low-intensity sonication, interspersed with 30 s ice-bath submersion. The suspension was centrifuged at 2772*g*, for 10 min at 4 °C to eliminate unbroken cells. Membranes were pelleted by ultracentrifugation of supernatant at 100 000 *g*, at 4 °C, for 60 min. Membranes were resuspended in TGE buffer (75 mmol/l TrisHCl. 10 % (v/v) glycerol, 25 mmol/l EDTA, pH 7.5) using a Dounce homogenizer and stored at -80 °C. Protein concentrations were determined using the method described by Bradford, following the manufacturer's protocol from BioRad (San Francisco, California, USA), using bovine serum albumin as standard.

CYP and b_5 concentrations in bacterial cultures and membrane preparations were determined using spectrophotometric techniques as described before [38, 42]. The CYPOR content of membrane preparations was determined using the NADPH-cytochrome *c* reduction assay as described previously [40].

Western blot analyses were carried out as described previously [18], except for the use of polyclonal (rabbit) antiserum for the detection of b_5. Determination of the activity parameters of clozapine *N*-demethylation, phenacetin *O*-deethylation, 7-methoxy-, 7-ethoxy-resorufin *and* 3-cyano-7-ethoxycoumarin *O*-dealkylation, α-NF inhibition as well as the bioactivation (mutagenicity) assays and multivariate statistical analysis were performed as described previously [18].

3.3. RESULTS

The *E. coli* cell model, BTC [38], was used for the separate coexpression of the human CYP1A2 variants together with both human CYPOR and b_5 using a biplasmid

system [33, 38]. In addition, a CYP1A2 null strain expressing only human b_5 with CYPOR was constructed (BTCb_5Por) for use as control to verify that catalytic alterations are because of the combined effect of b_5 with the CYP1A2 variants, and not related to independent b_5 metabolism.

CYP and b_5 concentrations in bacterial cultures were determined (see Figures, Supplemental digital content 2 and 3, *http://links.lww.com/FPC/A539,* which shows the CYP and b_5 contents in whole cells). The b_5 holoprotein concentrations determined in whole bacterial cells were comparable among all strains. The CYP holoprotein levels of almost all the variants were similar to WT (ranging from 157 to 231 nmol/l) except for variants I386F and R456H. The CYP levels of mutant I386F were significantly lower than those of WT, and R456H did not show any detectable holoprotein when determined in whole bacterial cells.

Immunoblot analysis of microsomal preparations indicated that both CYP and b_5 protein expression levels were similar between all strains (see Figures, Supplemental Digital Content 4 and 5, *http://links.lww.com/FPC/A539,* which shows CYP1A2 and b_5 immunoblot analysis of microsomal preparations). CYP, CYPOR, and b_5 contents were determined in microsomal membrane fractions (Fig. 1). CYPOR and b_5 contents ranged from 6 to 9 pmol/mg and 7 to 14 pmol /mg protein, respectively, being comparable among all strains. The CYP holoprotein amount was relatively similar to WT in membrane preparations for variants S212C, S298R, G299S, I314V and C406Y (ranging from 68 to 105 pmol /mg protein) whereas variant T83M (55 pmol /mg protein) showed a slightly lower level. CYP1A2 variants I386F and R456H did not show any detectable CYP holoenzyme in their membrane preparations, as was the case in our former study [18], probably because of inefficient heme incorporation and/or anchoring as WT-like levels of apoprotein were determined by immunodetection for these two variants (see Figures,

Supplemental Digital Content 4 and 5, *http://links.lww.com/FPC/A539,* which shows CYP1A2 and b_5 immunoblot analysis of microsomal preparations).

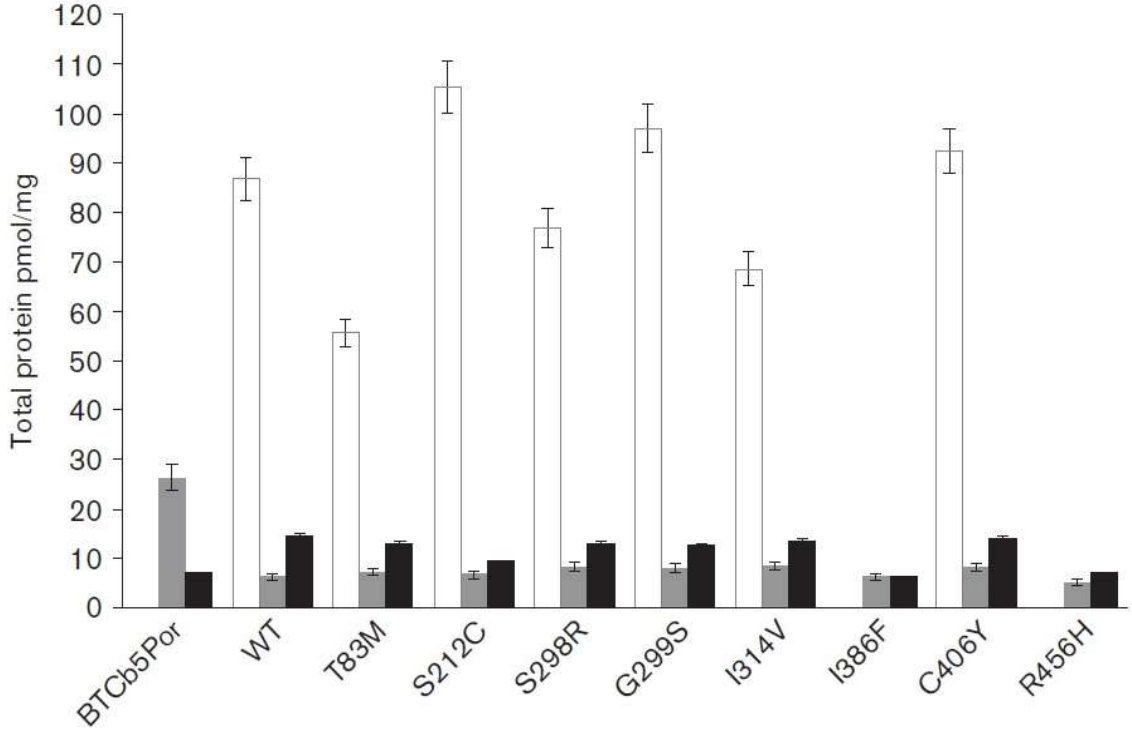


Figure 1. CYP (white bars), b_5 (solid bars) and CYPOR (grey bars) contents of microsomal membrane fractions (pmol/mg total protein).

The CYPOR/CYP ratios (0.06-0.13) for the different strains were in the range of values observed in human liver microsomes (0.08-0.5) [43]. The b_5/CYP ratios were calculated (0.09-0.23) and were a fraction lower when compared with those observed in human liver microsomes (0.27-2.70) [44]. Both CYP1A2 and CYPOR expression levels were similar to those obtained in our former study [18], that is when coexpressed without b_5.

3.3.1. Enzyme activities of CYP1A2 variants in the presence of cytochrome b_5

To determine enzyme activities of all CYP1A2 allelic variants, co-expressing both human b_5 and CYPOR, eight structural different substrates were used and their activities were compared with the WT variant (see Figure, Supplemental Digital Content 6, *http://links.lww.com/FPC/A539,* which shows the chemical structures of the substrates used). Three fluorogenic CYP1A2 substrates (ER, MR and CEC), and two therapeutic drugs (clozapine and phenacetin) were used. Furthermore, the bioactivation of three CYP1A2 dependent premutagens (2AA, IQ and NNK) was measured. Results of all activity measurements are summarized in Table 1.

3.3.1.1. Kinetic studies with fluorogenic substrates

The polymorphic CYP1A2 variants and its WT form were tested with three typical CYP1A2 fluorogenic substrates (ER, MR and CEC). The results of the Michaelis-Menten kinetic studies (yielding V_{max} and K_M values) as well as the inhibitory potency (IC_{50}) of α-NF, a specific CYP1A inhibitor, are presented in Table 1. Although K_{cat} (V_{max}/K_M) is considered to be a valid kinetic descriptor, subtle but significant differences in K_M or V_{max} can be obscured when only using K_{cat} values. As such, K_M and V_{max} were carried forward separately, as in our former study [18]. Surprisingly, variant I386F, which showed no (spectrophotometrically) detectable holoprotein in membrane fractions, showed activity for the three fluorogenic substrates. Membrane preparations of this variant were applied using the same total protein concentration as that for the WT variant, which showed equal apoprotein levels in the immunodetection (see Supplemental Figure 4, *http://links.lww.com/FPC/A539*). This activity was not because of any CYP1A2-independent metabolism as membranes derived from the negative control strain

(BTCb_5Por) did not show any activity with these three substrates. For variant R456H also lacking detectable holoprotein, no activity could be measured for any of these three substrates, not even at a double-membrane sample concentration and at increased substrate concentrations.

Variants T83M, I314V and C406Y showed a small increase in V_{max} compared with WT, for both MR and ER, but not for CEC. Only variant T83M showed the same pattern of increase in V_{max} for CEC. However, the affinity for these three substrates was not significantly altered; only variants I314V and C406Y showed a small decrease in K_M comparing with WT for CEC. Variants S212C, S298R and G299S showed activity parameters comparable with the WT variant for these three substrates, except for variant S212C [V_{max} (MR) less than half of the one found for WT], variant G299S [K_M (ER) slightly lower than the one found for WT] and S298R [V_{max} (MR) and K_M (MR) slightly lower than those of WT].

Finally, variant I386F, which, in our former study, did not show any activity for these substrates, not only showed activity in the presence of b_5 as mentioned above but also showed different types of effects between substrates. The apparent affinity of this variant for these three substrates was decreased (higher K_M values) when compared with those of the WT variant. This was also the case for the maximum reaction velocity (V_{max}), which was about half (MR) or less (CEC) than the ones determined for the WT variant, except for the substrate ER. This substrate was metabolized at a doubled velocity by variant I386F compared with the WT variant.

3.3.1.2. Kinetic studies with clozapine and phenacetin

The clozapine *N*-demethylation and phenacetin *O*-deethylation activities of the CYP1A2 variants were determined, as in our previous study [18]. On the basis of the

kinetic analysis of WT, two substrate concentrations were chosen: one resembling the apparent affinity concentration (K_M) and one well beyond the concentration point where V_{max} was reached. Membranes of variant I386F showed no activity with clozapine and only (low) activity with the higher concentration of phenacetin. Again, R456H showed no activity for any of the two substrates, even at the highest substrate and double-membrane concentrations.

The seven remaining variants and WT were incubated with 50 and 250 µmol/l of clozapine (Table 1 and see Figure, Supplemental Digital Content 7, *http://links.lww.com/FPC/A539,* which shows the clozapine N-demethylation of CYP1A2 variants), and 20 and 200 µmol/l of phenacetin (Table 1 and see Figure, Supplemental Digital Content 8, *http://links.lww.com/FPC/A539,* which shows the phenacetin O-dealkylation of CYP1A2 variants). All control assays were carried out in the presence of 1 mmol/l of the CYP1A2 inhibitor α-NF with the highest concentration of each substrate.

In the case of clozapine *N*-demethylation using the lower substrate concentration, all variants showed an overall decreased activity when compared with WT, whereas this decrease was less apparent at the higher concentration. With 20 µmol/l clozapine, variants T83M, S212C, S298R, G299S, I314V and C406Y showed, respectively 38, 64, 77, 82, 56 and 75% of the N-demethylation velocity found for the WT variant. In the case of 200 µmol/l clozapine, these were 91, 67, 91, 75, 114 and 88%, respectively, of the WT activity.

For both phenacetin substrate concentrations, the *O*-deethylation activity of all variants showed an overall decreased activity when compared with WT. When using 50 µmol/l substrate, variants T83M, S212C, S298R, G299S, I314V and C406Y showed reaction velocities of 30, 62, 80, 111, 71 and 47% of WT, respectively. These velocities were, respectively 70, 60, 75, 71, 87 and 74% of the WT activity when measured with a

phenacetin concentration of 250 μmol/l . In the case of I386F, which only showed activity using the higher concentration of phenacetin, the reaction velocity represented ~6% of that found for the WT variant. The two negative control experiments (using 1 mmol/l of the CYP1A inhibitor α-NF with the WT variant or the null CYP1A2 strain BTCb_5Por), did not show any activity for these two substrates, as expected.

3.3.1.3. Bioactivation of premutagens

The BTC strains, coexpressing recombinant human CYP1A2, CYPOR and b_5, have previously been shown to be an efficient and appropriate system to study bioactivation of several known CYP1A2-dependent mutagens [38]. The role of b_5 in the bioactivation activities of the eight polymorphic variants of CYP1A2 was evaluated with three known procarcinogens, namely, 2AA, NNK and IQ (Table 1 and see Figures, Supplemental Digital Content 9-11, *http://links.lww.com/FPC/A539,* which shows the mutagenic activities toward 2AA, IQ and NNK of the CYP1A2 variants). The three compounds were also assayed using the CYP1A2 null strain (BTCb_5Por), which did not show any bioactivation capacity (mutagenicity) for the dose ranges applied for all CYP1A2 variant strains.

Strains expressing variants T83M and C406Y showed a significant decrease in mutagenic activity when testing 2AA (52 and 64%, respectively) compared with the strain expressing the WT variant, whereas strains expressing all other variants were not significantly hampered in bioactivating 2AA. In the case of IQ, the strain expressing variant I386F could not bioactivate this carcinogen. Strains containing variants T83M and C314V showed a significant decrease in mutagenic activity, representing ~47 and 54% of the activity of the strain with CYP1A2-WT. Again, strains containing all other variants showed no significant differences compared with the CYP1A2-WT strain. The ability of

variants T83M and S212C to bioactivate NNK was markedly decreased, showing ~19 and 50% of the activity of the WT variant. All other variants showed no significant differences compared with WT for this carcinogen. The strain expressing variant R456H, one of the two CYP1A2 variants with perturbed expression of holoenzyme, showed no mutagenic activity for any of the three premutagens.

3.3.2. Multivariate analysis of activity data

The experimental data set (Table 1) was combined with the experimental data set of our previous report on all CYP1A2 variants [18], that is the activity parameters of all CYP1A2 variants were obtained in the same way, but were expressed without b_5. Multiple multivariate analyses were carried out to examine the effect of b_5 objectively. The procedure for data normalization was based on the variance in the data set. Normalization by variance was applied both by column and by row. This type of double normalization is appropriate when different enzyme preparations are being compared, and different types of substrates and detection methods are used [45]. It is important to note that both data sets, the data from this present study and the one from our previous study [18], were combined and analysed as a single data set and not compared with each other as different data sets. Data of variants R456H and I386F were excluded from the multivariate analysis as R456H had no detectable activities for any of the assayed substrates, and I386F only showed activities in this presence of b_5 (i.e. present study), both considered statistically perturbing for this type of analysis.

The first analysis of comparison of the CYP1A2 variants involved the direct hierarchical classification of the data set on the basis of the Ward method with complete linking (Fig. 2). It seems clear that the tendency of the variation in the combined data set is mainly ruled by the presence or absence of b_5. In the absence of b_5, CYP1A2 variants

G299S and T83M behave most differently from the WT variant, corroborating the results of our previous analysis [18]. In the presence of b_5, variant T83M is the clear outlier, with variants S212C and C406Y being slightly different from WT. Subsequently, a clustering by similarity was carried out using the Pearson correlation with average linkage, an analysis that determines which of the measured activity parameters was more powerful in showing altered biotransformation capacities among the CYP1A2 variants. A dendogram on the basis of the normalized data set was obtained (Fig. 3) and NNK bioactivation capacity was segregated from all other activity parameters, as was found in our former study [18].

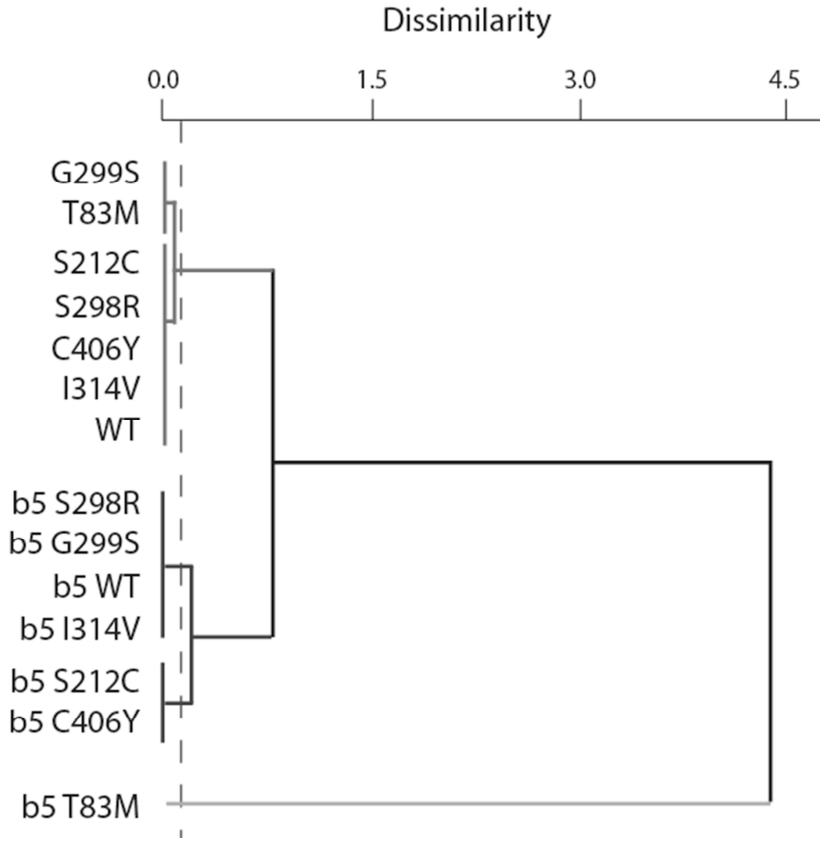


Figure 2. Dissimilarity tree for the different CYP1A2 variants. This classification was obtained directly from the data set based on the Ward method with complete linking.

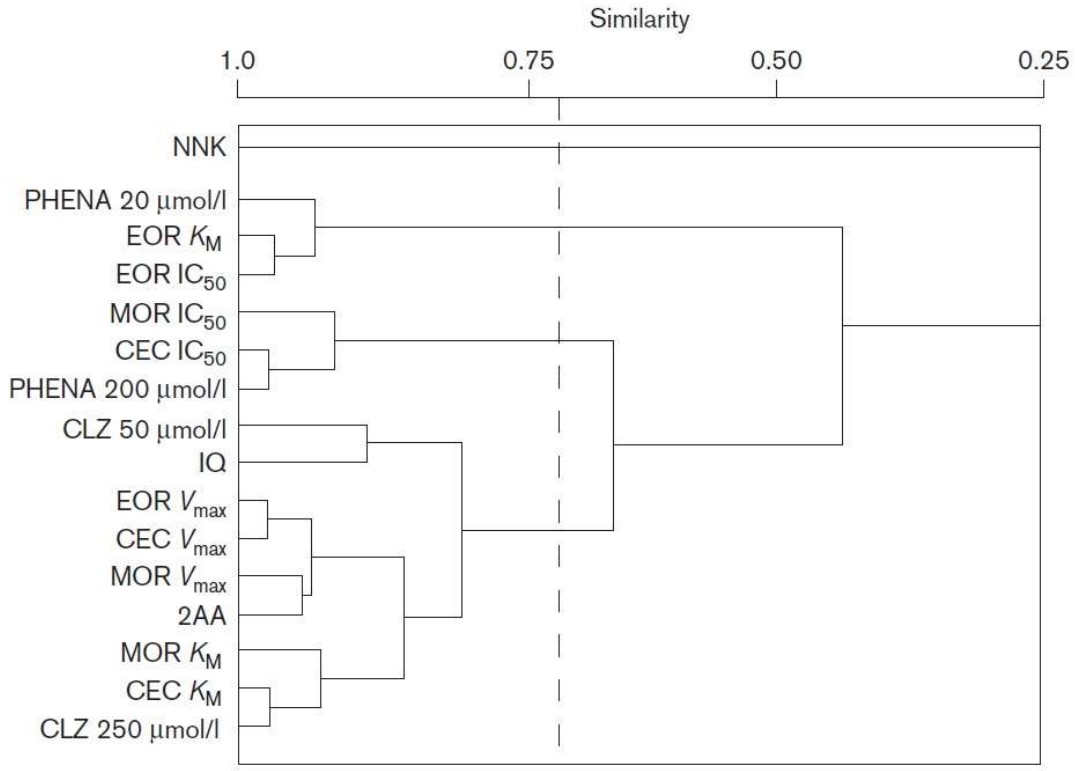


Figure 3. Clustering performed by similarity using the Pearson correlation with average linkage, for all the different activities.

3.3.2.1. Principal component analysis

The principal component analysis (PCA) [46] was used to project the data set from a 16-dimensional (activity parameters) space into a two-dimensional space for ease of visualization (Fig. 4). The trend of variation that governs the merged activity data set stems from the difference between variantactivities in the presence or absence of b_5, and this analysis retains 91% of the initial variance [principal component 1 (67%) plus 2 (24%)], which indicates a high significance in the distribution of the CYP1A2 variants over the b_5(+) and b_5(-) clusters. Variant G299S appears apart from the remaining WT-like enzymes in the b_5(-) cluster, with variant T83M slightly but significantly separated, corroborating the results of our previous study [18]. In the case of the b_5(+) cluster, the T83M variant of CYP1A2 is now completely segregated, together with variant C406Y from all other variants that are more WT like. Furthermore, it seems that variants

aggregate closer together (more WT like) in the b_5(+) cluster compared with those of the b_5(-) cluster.

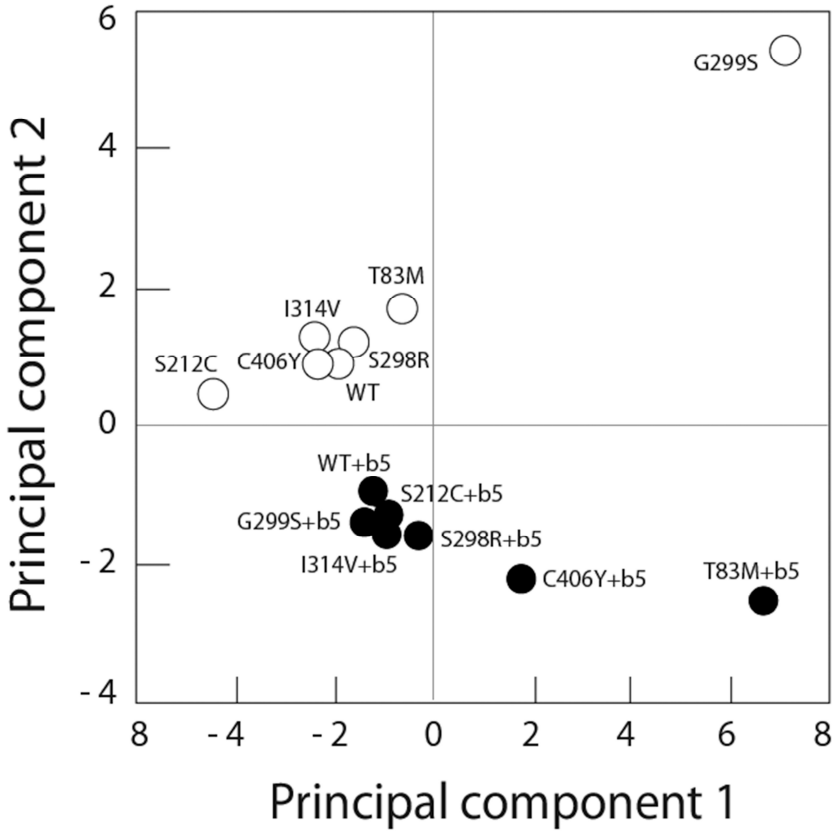


Figure 4. PCA plot of the CYP1A2 variants' (with and without b_5) activity- normalized data set for the first two principal components (91% of the initial variance retained).

Table 1. Data set of all measured activity parameters of the CYP1A2 variants when coexpressed with CYPOR and cytchrome b_5.

	MROD V_{max} (pmol R/min)		MROD K_M (µmol/l)		EROD V_{max} (pmol R/min)		EROD K_M (µmol/l)		CECOD V_{max} (µmol/l CHC/min)		CECOD K_M (µmol/l)		Phenacetin 20 µmol/l (pmol paracetamol/min)		Phenacetin 200 µmol/l (pmol paracetamol/min)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
WT	1.17	0.043	0.61	0.055	0.48	0.012	0.30	0.028	3.81	0.100	2.64	0.306	6.32	0.38	255.6	6.7
T83M	1.94	0.031	0.68	0.025	0.87	0.020	0.38	0.031	5.52	0.099	2.40	0.195	1.87	0.33	178.9	1.4
S212C	0.49	0.014	0.54	0.039	0.41	0.010	0.25	0.024	3.07	0.092	2.57	0.339	3.91	0.12	153.6	10.7
S298R	0.88	0.020	0.43	0.026	0.44	0.013	0.25	0.029	3.63	0.128	3.48	0.511	5.04	0.35	191.8	2.2
G299S	0.96	0.024	0.50	0.032	0.57	0.019	0.21	0.028	3.04	0.099	2.32	0.345	7.04	0.17	181.1	6.6
I314V	1.75	0.052	0.59	0.043	0.84	0.023	0.30	0.031	4.50	0.112	1.95	0.229	4.47	0.18	222.3	9.3
C406Y	1.61	0.083	0.74	0.087	0.74	0.031	0.40	0.059	3.35	0.096	1.94	0.263	3.00	0.12	189.1	8.5
I386F	0.59	0.011	0.76	0.032	0.83	0.035	0.58	0.077	1.16	0.026	4.13	0.364	--	--	15.8	0.4

	Clozapine 50 µmol/l (pmol N-demethylation product/min)		Clozapine 250 µmol/l (pmol N-demethylation product/min)		2AA (rev/nmol)		IQ (rev/nmol)		NNK (rev/µmol)		IC_{50} (α-NF) MROD[a] (µmol/l)	IC_{50} (α-NF) EROD [a] (µmol/l)M	IC_{50} (α-NF) CEC [a] (µmol/l)M
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SDv			
WT	51.4	0.0	83.9	1.4	7611	534	409	52	2707	393	0.020	0.063	0.199
T83M	19.5	0.9	76.7	3.4	3958	192	192	12	522	12	0.033	0.053	0.160
S212C	32.8	0.7	56.4	2.2	7199	623	358	37	1353	95	0.033	0.065	0.144
S298R	39.4	0.3	76.3	4.4	6584	427	344	43	2334	372	0.018	0.035	0.202
G299S	42.3	0.7	63.0	2.9	7499	386	304	45	3211	105	0.025	0.030	0.232
I314V	28.8	0.2	96.0	2.8	7354	681	221	47	2304	316	0.018	0.037	0.313
C406Y	38.5	0.4	74.1	0.5	4874	298	317	77	1963	113	0.018	0.025	0.162
I386F	-	-	-	-	6759	325	-	-	2253	28	0.036	0.073	1.399

(-): no activity measurable. [a]: IC_{50} values with a 95% confidence interval, calculated by a sigmoidal dose-response analysis using GraphPad Prism 5 software (GraphPad Software, San Diego, California, USA)

3.4. DISCUSSION

Genetic polymorphism in xenobiotic metabolizing enzymes is currently accepted as one of the main factors in the interindividual differences observed in drug and xenobiotic responses and susceptibilities. CYP enzymes are affected by various environmental, individual habits, and genetic factors, which make drug metabolism highly variable and even almost individualistic [47]. The CYP1A subfamily comprises two members, CYP1A1 and CYP1A2, with the latter being almost exclusively expressed in the liver. Genetic factors were suggested to represent 35-75% of the interindividual variability for CYP1A2 activity [48, 49]. Furthermore, the number of clinical drugs effecting CYP1A2 activity is increasing and the opportune identification of these drug interactions is important both in clinical practice and in drug discovery and development [50].

To carry out catalysis, CYPs require two electrons, which are normally delivered by CYPOR. Another heme protein, b_5, has been shown, at least for three decades, to alter the rate of catalysis by specific CYPs [32, 36, 51]. It has been reported to enhance, inhibit, or not to alter the catalysis of CYP, depending on the substrate, the CYP isoform, and the experimental conditions [37]. Despite the substantial work on the function and effect of b_5 on CYP-mediated catalysis in the last decades, its mode of action is still not fully understood. Collectively, these studies, and in particular the work of Dr Lucy Waskel's group (reviewed in Im and Waskell [52]), seem to indicated that (i) in principle, the first electron can only be donated by CYPOR, (ii) b_5 can donate the second electron in competition with CYPOR, (iii) CYPOR and b_5 donate the second electron at a similar speed in the formation of the reduced oxyferrous CYP, and (iv) the decay to the ferric ground state of CYP plus product release is accelerated when the second electron is delivered by b_5 [52]. This acceleration of the actual oxygenation step of the substrate was

hypothesized to be caused by b_5-mediated induction of conformational changes at CYP's active site [52].

To our knowledge, so far, no study has been reported on the systematic evaluation of the effect of b_5 on human CYP polymorphic variants. As such, the goal of this study was to examine the effect of human b_5 on the activity of eight polymorphic variants and WT of human CYP1A2 using a set of eight structurally diverse substrates.

For this purpose a bacterial *E. coli* cell model, which does not contain any endogenous (bacterial) CYP or b_5, was applied for the coexpression of the CYP1A2 variants with CYPOR and b_5. The CYP contents were similar to WT for all variants, except for I386F and R456H. Although these two variants showed similar levels of CYP1A2 through immune detection (apoprotein), neither showed detectable holoenzyme in membrane fractions or in whole bacteria except for variant I386F (markedly reduced levels in whole cells). This indicated highly unstable holoenzymes of these variants (even in the presence of b_5), corroborating our previous results [18]. Both CYPOR and b_5 expression levels in membrane preparations were similar in all strains. Comparing CYP levels with those of our former report [18], coexpression of the CYP1A2 did not seem to be influenced by the coexpression with b_5 as no significant deviations could be observed. Both CYP1A2 and CYPOR expression levels were similar to those obtained in our former study [18] when coexpressed without b_5. Furthermore, CYPOR/CYP ratios for the different variants were in the range of those observed in human liver microsomes, whereas the b_5/CYP ratios were a fraction lower than the ones observed in human microsomes. Several in-vitro studies have been reported on the dose effect of b_5, describing increasing effects when applying increased b_5 levels (reviewed in Im and Waskell [52]). As such, the effects of b_5 presented in this current study will be expected to be more pronounced *in vivo*. Still, with respect to the main goal of our study, the

variations observed in CYP1A2 activity can be uniquely attributed to the CYP1A2 variant itself and/or the presence/absence of b_5.

CYP1A2-WT and its variants were characterised for 16 different activities parameters using structurally different substrates, representing different types of CYP1A2-mediated reactions, specifically: *N*-demethylation (clozapine); *O*-dealkylation (MR, ER, CEC and phenacetin); and *N*-hydroxylation (NNK, IQ and 2AA). As this characterization was carried out in the same way as in our former report [18], the current data set could be combined with the one of our previous study [18]. This combined data set was examined using several multivariate analysis tools to analyse all activity data to objectively delineate the effect of b_5 on the CYP1A2 variants. The combined data set was analysed by PCA [46], a very useful statistical tool of complementary importance as shown in several fields [53, 54] , plus the recent exploration of the protein structure space [55, 56].

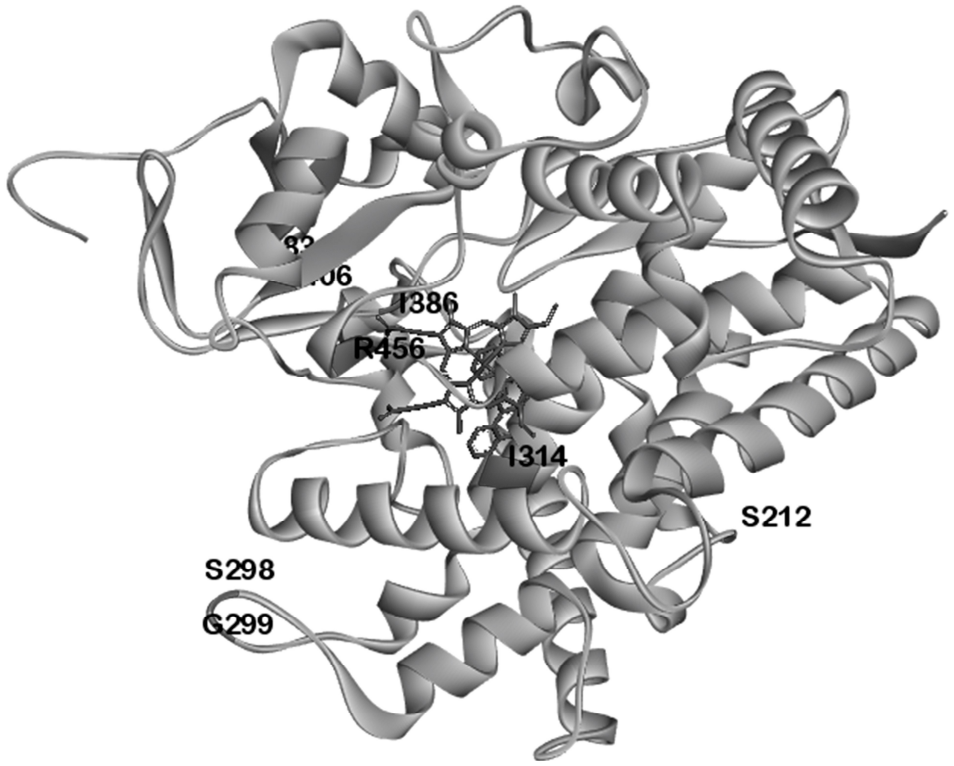
Both the Ward Method and the PCA (Figs 2 and 4) showed that all CYP1A2 variants behaved quite distinguishably in their activity in presence of b_5 compared with their activities in absence of b_5, forming two separated clusters. Both the PCA and the Ward Method corroborate our previously published results [18], in which the nine CYP1A2 variants were analyzed for the same activity parameters in absence of b_5. In this analysis, variant G299S was the most altered CYP1A2 enzyme relative to the WT variant, whereas T83M was slightly but significantly different from WT. Conversely, variant G299S was closely related with the WT form of CYP1A2 both in the PCA as well in the dissimilarity tree, when assayed in the presence of b_5, whereas the more deviated forms were variants T83M and C406Y, consistently with both the PCA and Ward analysis method.

The PCA of the variants indicates which amino acid substitutions in the protein are related to activity profile alterations of CYP1A2 and can thus be informative of the functionalities of the protein structure. On the basis of the human CYP1A2 crystal structure (PDB #2HI4) published by Sansen *et al.* [39] (Fig. 5a and b) and from the description of substrate entrance and product exit channels of CYPs by Cojocaru *et al.* [57], residues T83 and C406 are located close to the substrate entrance/product exit channel. These two variants lacked detectable NNK bioactivation activity in absence of b_5 as described in our former report [18]. However, both T83M and C406Y showed NNK bioactivation in the present study, that is in presence of b_5, although less efficient compared with the WT variant. These results seem to suggest that b_5 can influence the structure of the substrate entrance/product exit channel and thus the substrate positioning at the active centre. For NNK, this seems to be particularly important as the Pearson correlation analysis indicated bioactivation of this substrate as the most powerful among the 16 measured activities on examination of CYP1A2 variants (Fig. 3), as was the case in our former study [18].

Variants I386F and R456H were found to be two particular variants with perturbed holoprotein stability, as was observed in our previous study. In the case of variant R456H, b_5 showed no influence in holoprotein stabilization, reinforcing the importance of this residue in heme anchorage as was concluded in our previous report [18]. Residue R456 is located adjacent to C457, which is the conserved cysteine (fifth ligand) involved in heme binding [58] (Fig. 5a and b). The presence of b_5 was found to be crucial for the activity of variant I386F. In the absence of b_5, among the 16 measured activities, only (residual) 2AA bioactivation activities could be measured [18]. In the presence of b_5 (present study), I386F showed activity in most of the assays, with the exception of clozapine N-demethylation and IQ bioactivation (Table 1). The I386 residue is located in

the substrate recognition site 5 (SRS-5), which, together with the I-helix and the BC loop, represent the common structural features of the heme surroundings in mammalian CYP structures known to date [59]. The I386F substitution represents the replacement of an aliphatic side chain with an aromatic side chain, distorting the heme anchoring structure, explaining the highly unstable I386F holoprotein. Apparently, b_5 interacts with this variant, inducing a conformation that allows the (partial) stabilization of heme in the CYP1A2 protein, attenuating the detrimental loss of heme induced by the I386F substitution.

(a)



(b)

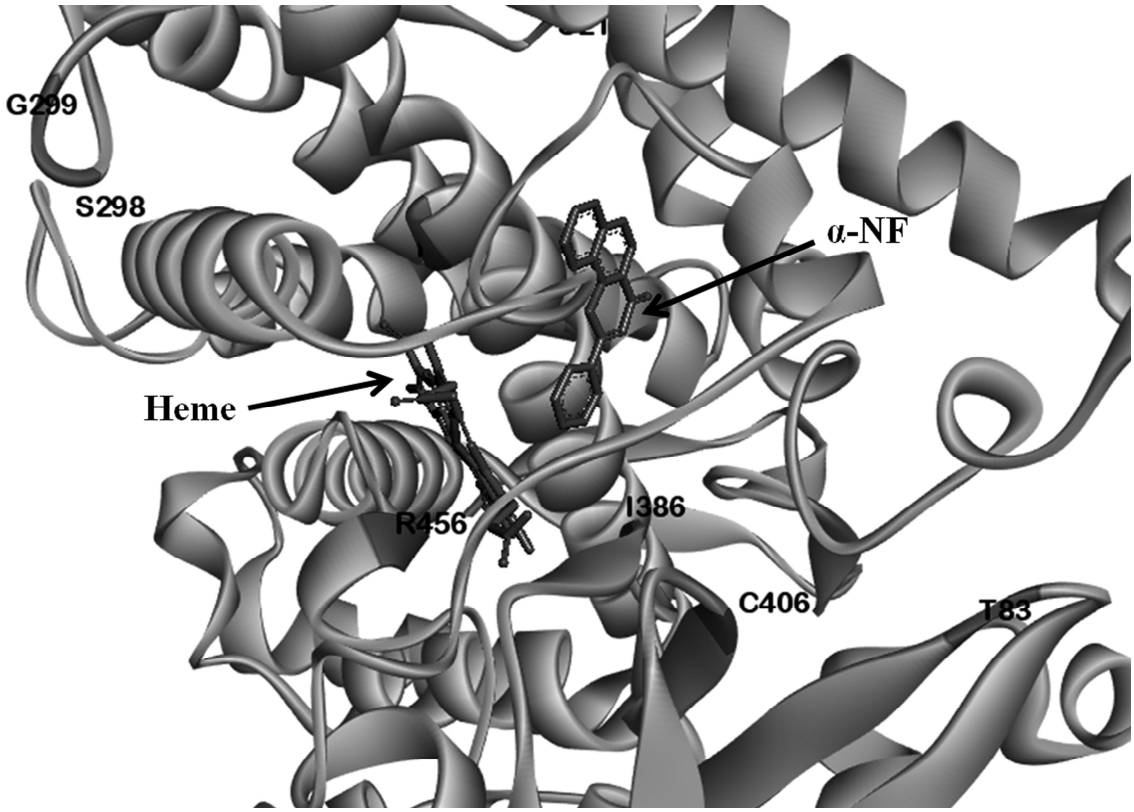


Figure 5. Localization of altered amino acids of the selected CYP1A2 polymorphic variants using the human CYP1A2 crystal structure published by Sansen, *et al* [39]. (a) Overview of CYP1A2 structure with the locations of mutations indicated; (b) Image zoomed in on the active center and the most relevant polymorphisms of this study, namely, residues T83 and C406, located close to the substrate entrance/product exit channel; residue R456 located adjacent to the fifth ligand involved in heme binding; residue I386 located in SRS-5 in the I-helix; and residues G299 and S298 located at the proximal side near the CYPOR/b_5 interaction zone. The prosthetic heme group as well as α-NF substrate are indicated. Figures were generated with the Discovery Studio v3.1 software (Accelrys, San Diego, California, USA),using PDB file # 2HI4.

The PCA showed striking results for variant G299S. This form is shown to be the most deviated variant in CYP1A2 activities in the absence of b_5, but was found to act as a WT-like enzyme in the presence of this facultative, auxiliary heme protein (Fig. 4). On the basis of the superposition and alignment of the human CYP1A2 crystal structure [39]

with the structure of the CYP BM3 heme and FMN-binding domains [60], residue G299 is located on the surface of the heme domain at the proximal side near the CYPOR/b_5 interaction zone (Fig. 5a and b). The secondary structural annotation of CYP1A2 (PDB #2HI4) also indicates that G299, along with S298, forms a β-hairpin structure. G299S replacement could be hypothesized to disrupt the local fold through steric hindrance of the β-hairpin. In contrast, S298R replacement could be hypothesized to be less disruptive to the β-hairpin, as evidenced by the similarity of S298R to WT in both the b_5(-) and the b_5(+) cluster of the PCA plot of catalytic data. Moreover, the G299 residue is located very close to the CYP1A2's C-helix, which is indicated to play an important role in the interaction with b_5 [52]. Previous reports of the group of Dr Lucy Waskel seem to indicate that both b5 and CYPOR interact with CYP on overlapping but nonidentical binding sites [32, 61]. On the basis of the "recovery" effect of b_5 with variant G299S (*vida supra*), it is tempting to speculate that residue G299 is part of this binding site on CYP1A2, which interacts exclusively with CYPOR.

This "recovery" effect of b_5 seems not to be exclusive for variants I386F and G299S. In the PCA analysis, most variants seem to group closer to the WT variant in the b_5(+) cluster (i.e. are more WT like) compared with the variants in the b_5(-) cluster (Fig. 4). CYP1A2 expression occurs almost exclusive in the liver, the organ where b_5 is unambiguously present. The apparent general attenuation in CYP1A2 activities of the genetic variants by b_5 might thus be an explanation for the lack of any clinical evidence to suggest that these particular genetic variants have phenotypic effects with respect to xenobiotic metabolism, and are as such considered to have low genetic penetrance of the CYP1A2 polymorphism to the phenotype level [62, 63]. Still, specific CYP1A2 alleles have been identified with altered drug clearance and response to drug therapy. However, these alleles showed genetic variation in the upstream, 5'-expression-regulating

sequences [50]. Variations in CYP1A2 activity can also be caused by the functional polymorphism of b_5 or CYPOR. Recently, genetic variants of b_5 have been described [64], although allelic variants are considered to be rare [65]. More than 50 different haplotypes of the human *POR* gene (encoding CYPOR) have been found so far (*http://www.cypalleles.ki.se/por.htm*). The effects of several CYPOR variants on human CYP1A2 have been reported by our group [40, 66].

Collectively, our data seem to indicate that b_5 exerts a compensatory effect on the perturbed functional capacities of the polymorphic variants of this study, which may be interpreted by b_5-induced allosteric attenuation of the conformational changes induced by their structural mutations. As such, b_5 seems to be capable of altering the conformation of CYP1A2 in a quite extensive manner, much more than only the active site architecture, the hypothesis postulated to explain the accelerated decay to the ferric ground state of CYP plus product release, when the second electron is delivered by b_5 relative to the case when this occurs by CYPOR [52] (see above). Indeed, our data on variant I386F (see above) seem to indicate the effect of $b_5$at the heme/substrate-binding site. However, our data on variants T83M and C406Y (substrate entrance/product exit channel; see above) are in particular indicative for the more extensive structural influence of b_5.

In summary, we successfully coexpressed the WT form and eight allelic variants of human CYP1A2 separately, together with human CYPOR and human b_5, and could measure 16 different activity parameters of the biotransformation of eight structurally different substrates. The heterogeneous data set generated from the current study was combined with the one of our previous study on these CYP1A2 variants [18], and analysed using several multivariate analysis tools. The general outcome of this analysis indicated the ability of b_5 to affect CYP1A2 variants to behave more like the WT variant, attenuating the detrimental effects of structural mutations of these variants, seemingly

through allosteric effects. This was particularly apparent for variant I386F, for which the presence of b_5 was crucial to show activity for the tested substrates. The results of this variant seem to corroborate the hypothesis that b_5 can accelerate CYP-mediated reactions through conformational changes at CYP's active site [52], as I386 is in close proximity to this center. However, the b_5-mediated recovery of the activity of variants T83M and C406Y, located considerably further from the active site, seem to indicate that this allosteric effect is much more extensive. Furthermore, our data seem to implicate CYP1A2 residue G299 in its interaction with CYPOR and/or b_5.

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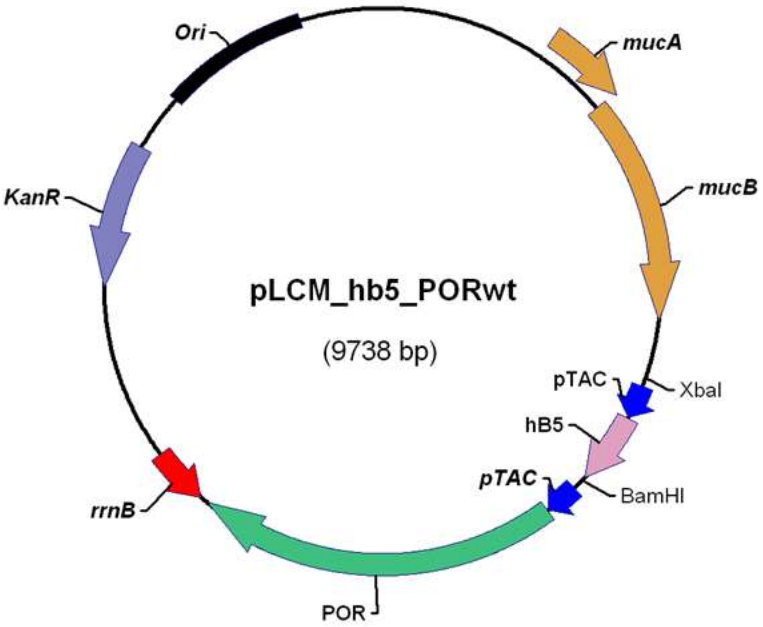
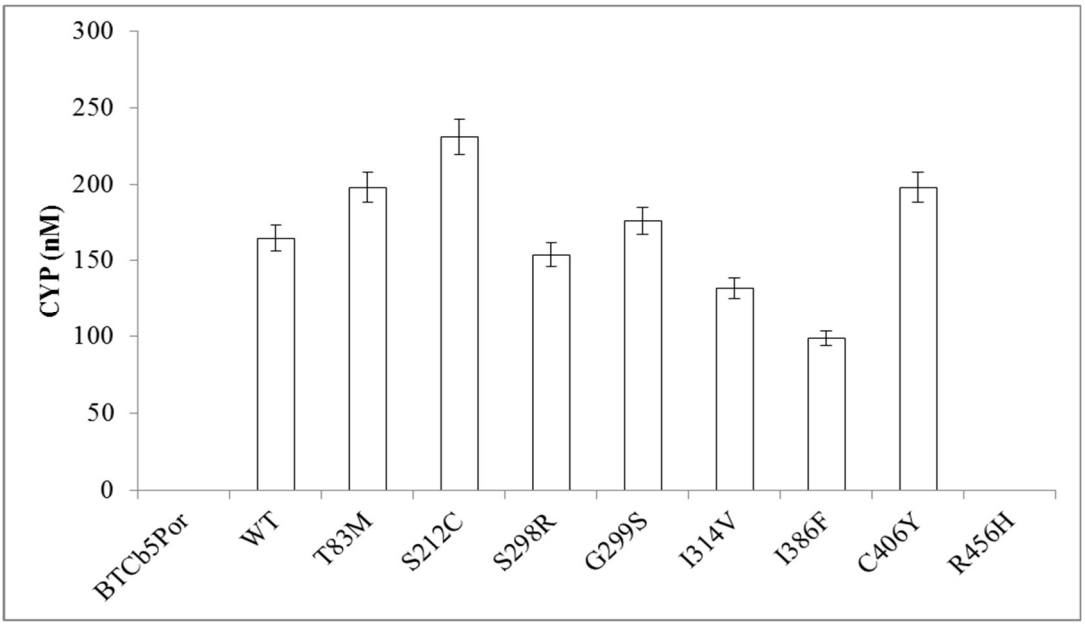
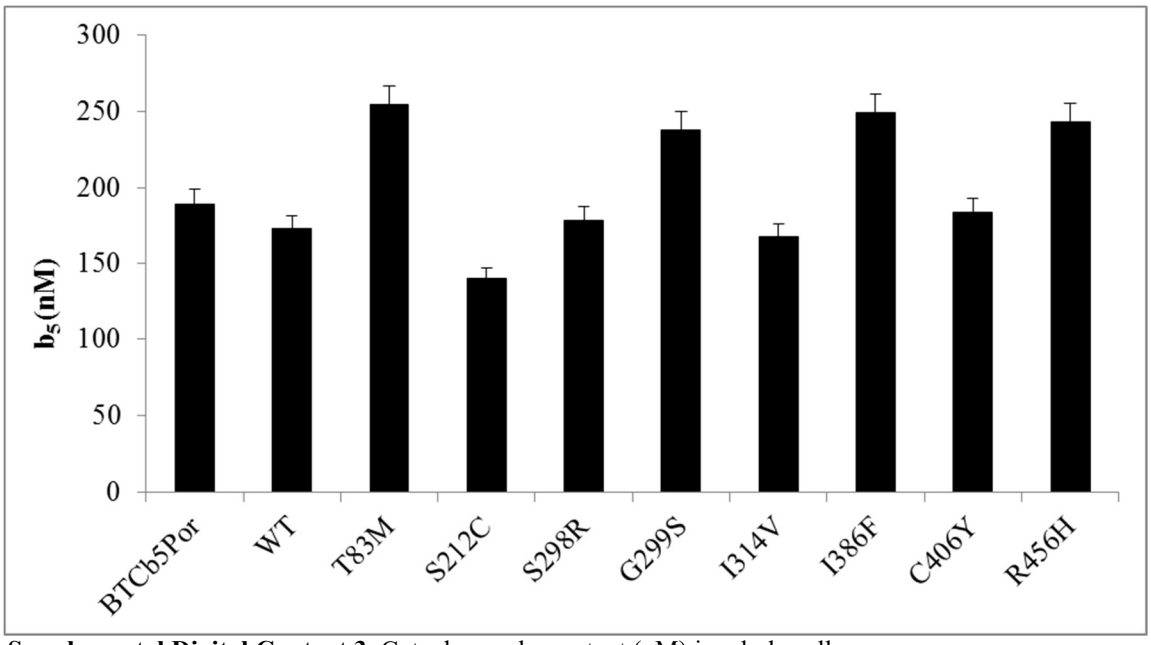
3.5. SUPPLEMENTAL DIGITAL CONTENT



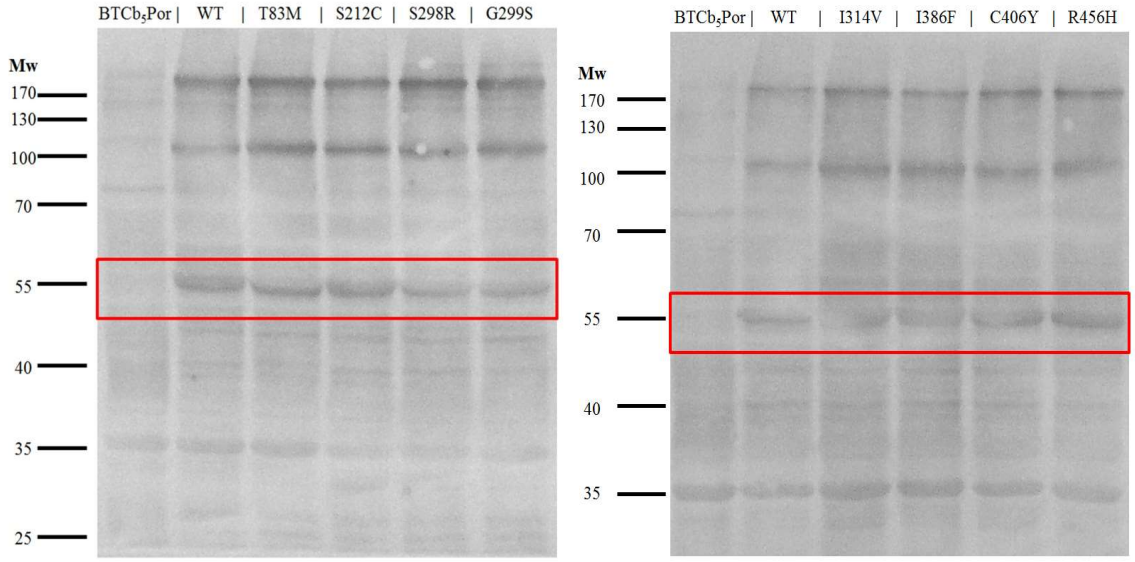
Supplemental Digital Content 1. Schematic representation of plasmid pLCM_hb$_5$_PORwt, used for the co-expression of human, full-length proteins b$_5$ and CYPOR with CYP1A2 variants.



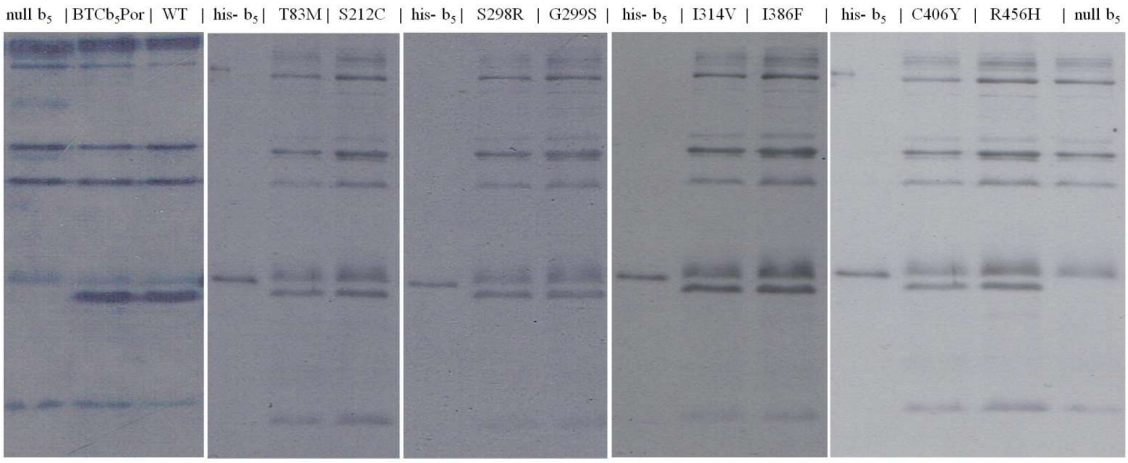
Supplemental Digital Content 2. CYP content (nM) in whole cells.



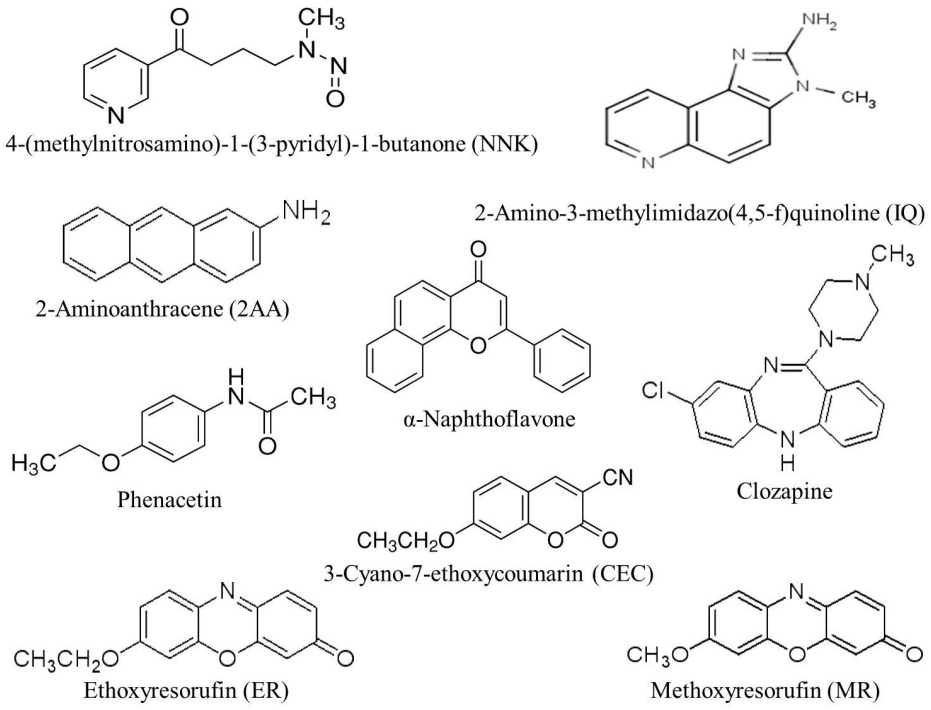
Supplemental Digital Content 3. Cytochrome b_5 content (nM) in whole cells.



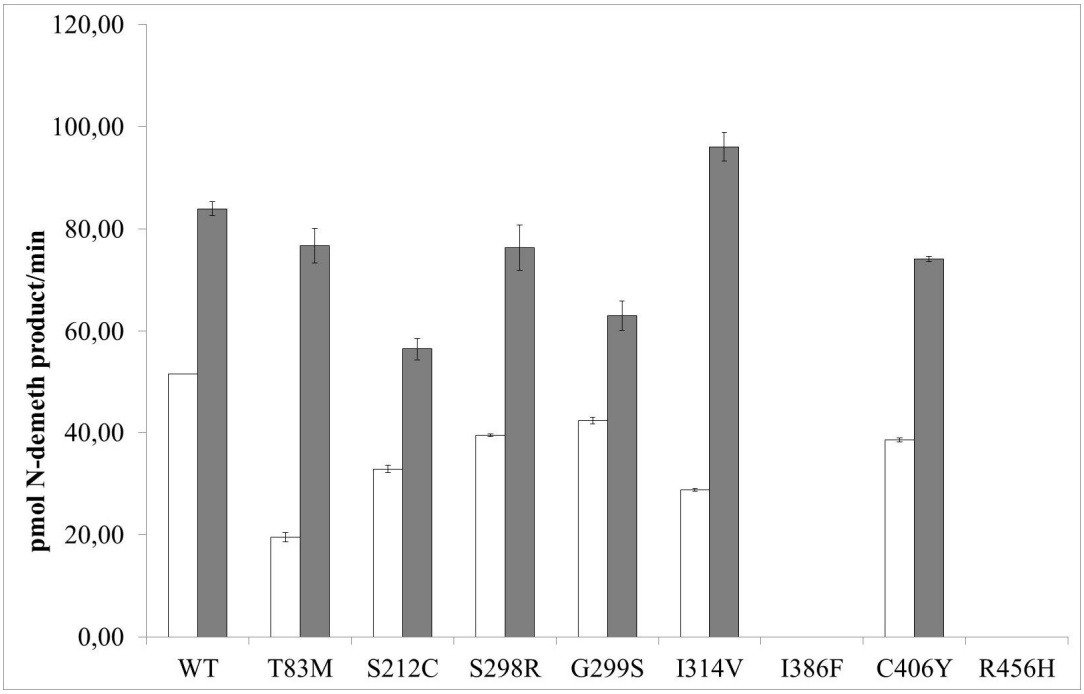
Supplemental Digital Content 4. Immunoblot analysis of CYP1A2 in *E. coli* stains co-expressing CYPOR, b_5 and CY1A2 polymorphic variants and WT. Each lane contained 10 µg of membrane fraction. Polyclonal (rabbit) CYP1A2-antiserum was used, demonstrating several non-specific signals, which can be easily distinguished through a comparison with the signals of the corresponding CYP1A2-null strain ($BTCb_5Por$) Mw: Molecular weight marker (values in kDa).



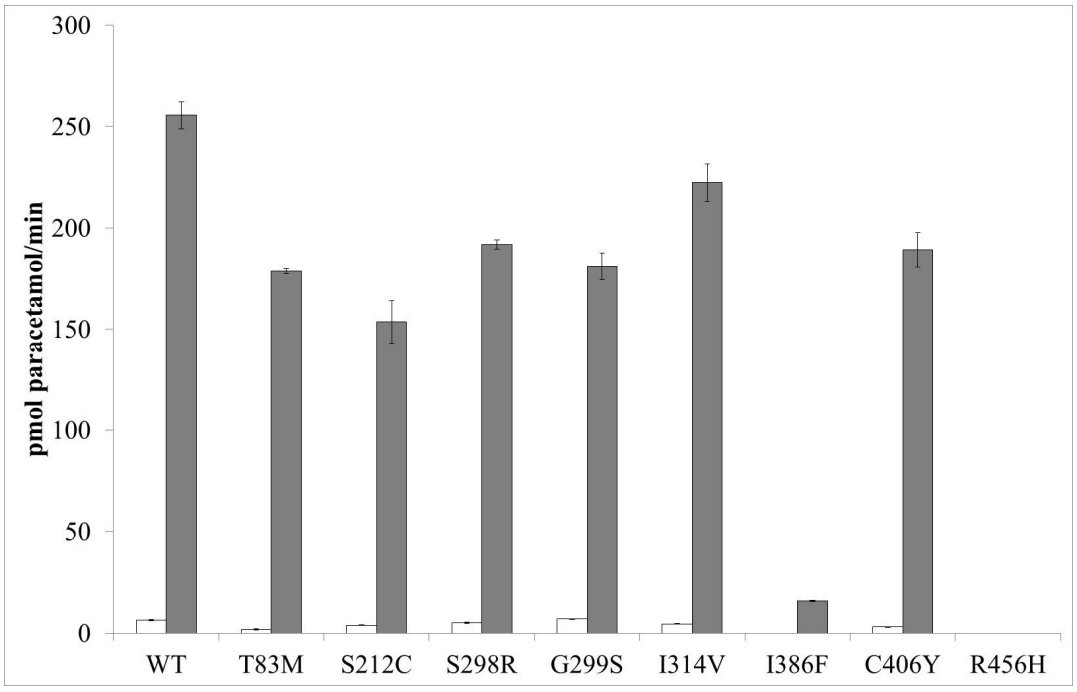
Supplemental Digital Content 5. Immunoblot analysis of b_5 in the BTC strains, co-expressing CYPOR, b_5 and CY1A2 variants. Each lane contained 10 µg of membrane fraction. Polyclonal (rabbit) b_5-antiserum was used, demonstrating several b_5-non-specific signals, which can be easily distinguished through a comparison with the signals of the corresponding b5-null strain. Purified his(6)-tagged b_5 was used as positive control, migrating slightly higher as the (non-tagged) b_5 in the BTC strains.



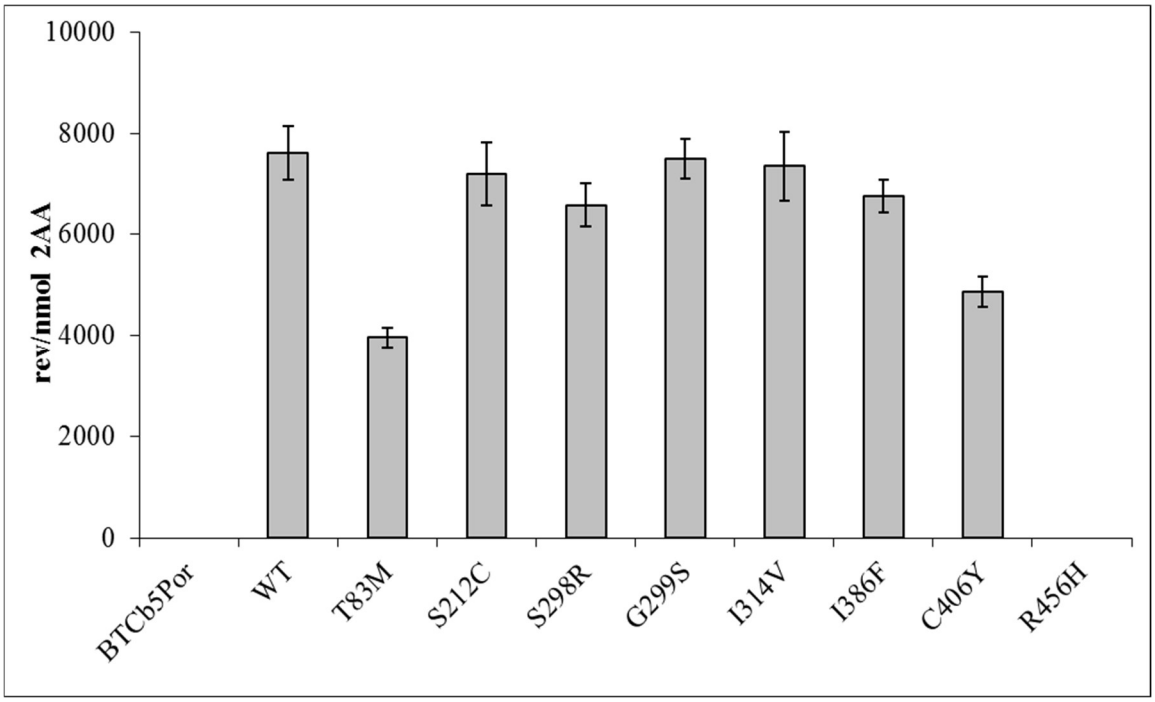
Supplemental Digital Content 6. Chemical structures of used substrates demonstrating the full structure-space of CYP1A2-compounds applied in this study.



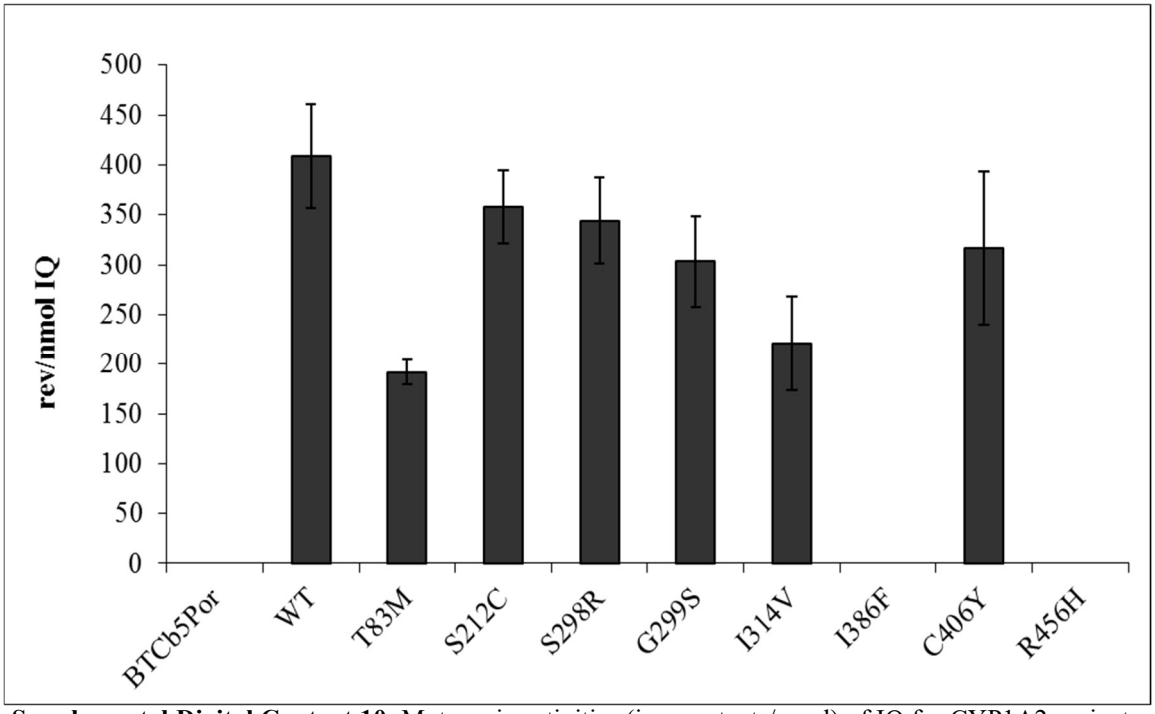
Supplemental Digital Content 7. Clozapine N-demethylation of CYP1A2 variants. All variants were assayed with 100 nM CYP. Relative activities determined with 50 µM (white bars) and 250 µM (grey bars) of clozapine.



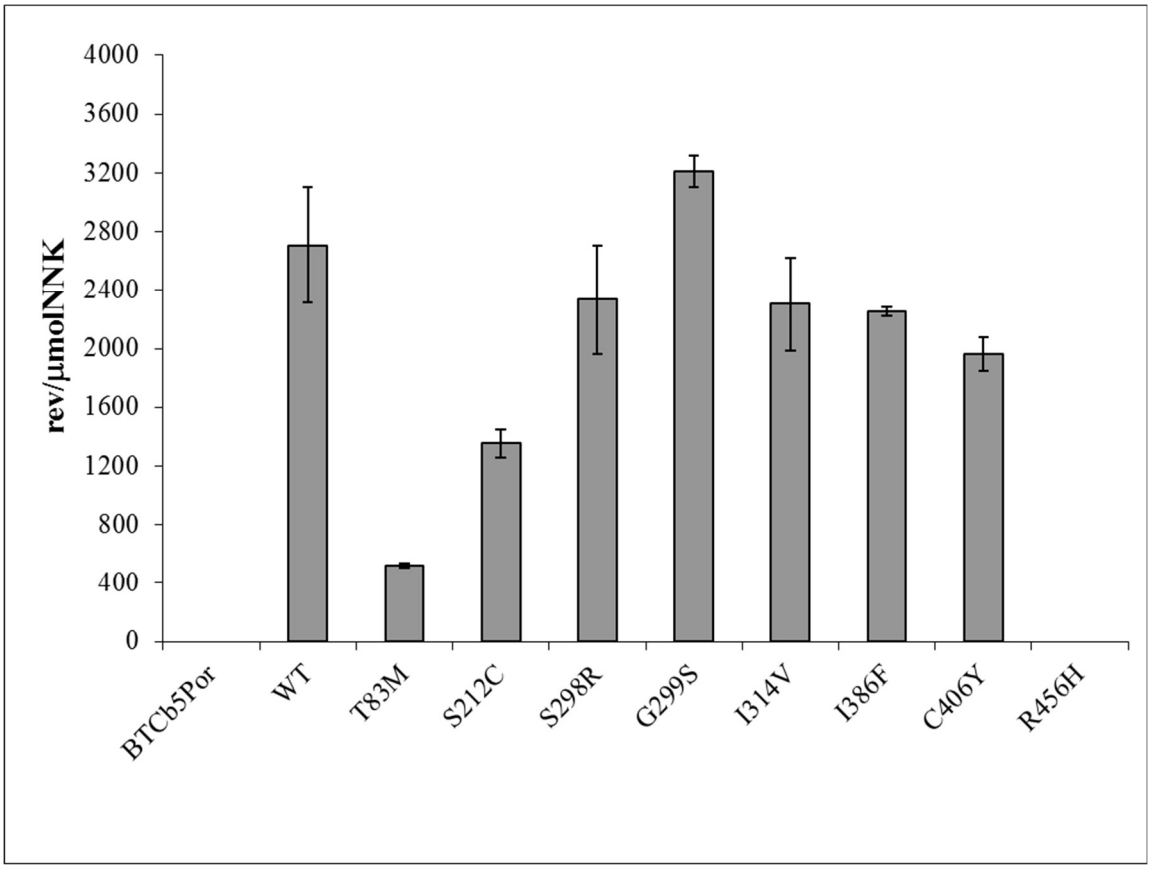
Supplemental Digital Content 8. Phenacetin O-dealkylation of CYP1A2 variants, when expressed in presence of b_5. All variants were assayed with 100 nM CYP. Relative activities determined with 20 µM (white bars) and 200 µM (grey bars) of phenacetin.



Supplemental Digital Content 9. Mutagenic activities (in revertants/nmol) of 2AA for CYP1A2 variants, when expressed in presence of b_5.



Supplemental Digital Content 10. Mutagenic activities (in revertants/nmol) of IQ for CYP1A2 variants, when expressed in presence of b_5.



Supplemental Digital Content 11. Mutagenic activities (in revertants/nmol) of NNK for CYP1A2 variants, in presence of b_5.

PART III

DEVELOPMENT OF BACTERIAL HTS-SYSTEMS FOR DETECTION OF GENOTOXIC ELECTROPHILIC REACTIVE METABOLITES

CHAPTER 4

Prototype systems containing human cytochrome P450 for high-throughput real-time detection of DNA damage by compounds that form DNA-reactive metabolites

Adapted from: Brito Palma, B.; Fisher, C.W.; Rueff, J.; Kranendonk, M.; *"Prototype Systems Containing Human Cytochrome P450 for High-Throughput Real-Time Detection of DNA Damage by Compounds That Form DNA-Reactive Metabolites"* Chem Res Toxicol. 2016 May 16; 29(5):747-56.

ABSTRACT

The formation of reactive metabolites through biotransformation is the suspected cause of many adverse drug reactions. Testing for the propensity of a drug to form reactive metabolites has increasingly become an integral part of the lead-optimization strategy in drug discovery. DNA reactivity is one undesirable facet of a drug or its metabolites and can lead to increased risk of cancer and reproductive toxicity. Many drugs are metabolized by cytochromes P450 in the liver and other tissues, and these reactions can generate hard electrophiles. These hard electrophilic reactive metabolites may react with DNA and may be detected in standard in vitro genotoxicity assays; however, the majority of these assays fall short due to the use of animal-derived organ extracts that inadequately represent human metabolism. The current study describes the development of bacterial systems that efficiently detect DNA-damaging electrophilic reactive metabolites generated by human P450 biotransformation. These assays use a GFP reporter system that detects DNA damage through induction of the SOS response and a GFP reporter to control for cytotoxicity. Two human CYP1A2-competent prototypes presented here have appropriate characteristics for the detection of DNA-damaging reactive metabolites in a high-throughput manner. The advantages of this approach include a short assay time (120-180 min) with real-time measurement, sensitivity to small amounts of compound, and adaptability to a microplate format. These systems are suitable for high-throughput assays and can serve as prototypes for the development of future enhanced versions.

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4.1. INTRODUCTION

Testing for the propensity of a compound to form reactive metabolites has increasingly become an integral part of lead-optimization in drug development [1]. To avoid costly late stage failures, predictive toxicology assays and models are being utilized as part of a 'fail early' or avoidance strategy [2]. Early detection of chemical liabilities by the identification of structures prone to form electrophilic intermediates through metabolism (structural alerts) can be used to mitigate safety issues. This is of particular importance when the chemically labile structures (toxicophores) might coincide with the pharmacophore and an early decision is needed to determine whether the pharmacophore is a good candidate for further study. Although in silico tools for identifying structural alerts are currently standard practice at the lead-optimization/candidate selection stage of drug development, it is imperative to demonstrate experimentally whether these predicted structural alerts are actually indicative of reactive metabolite formation [3]. Sensitive and efficient methods for detecting reactive metabolites are needed for evaluating drug candidates, thereby assisting medicinal chemists in optimizing a large number of lead compounds [4].

Hard electrophilic compounds that react with DNA might be detected in genotoxicity assays. DNA reactivity is an unwanted property of a drug or its metabolites due to the increased risk of cancer and reproductive toxicity [5]. Several different assays for in vitro genotoxicity screening are currently employed in drug development. These assays include a miniaturized version of the Ames mutagenicity test, the SOS-lux assay, VITOTOX, GreenScreen or BlueScreen, in combination with one or two cytogenetic tests (e.g., in vitro micronuclei test) [6-10]. The current standard in vitro approaches may fall short due to inadequate representation of reactive metabolites formed by human drug metabolism [11]. The major limitation of these standard tests in detecting genotoxicity of

human reactive metabolites is their reliance on a non-human (usually rodent liver extract) metabolic system to mimic human metabolism. Predicting drug toxicity in humans from results obtained in animals and animal-derived tissues is seriously hampered due to many species-specific differences in drug metabolism [12]. In particular, cytochrome P450, one of the major enzyme families in drug metabolism, has substantially different activities among species [12].

Over the years several genetic engineering approaches have been used by different laboratories for the development of bacterial genotoxicity reporter systems [13]. These systems rely on promoter-reporter fusions that generate a quantifiable dose-dependent signal in the presence of DNA-damaging compounds and the induction of repair mechanisms. The promoter, acting as the sensing element, regulates transcription of the downstream reporter gene, which can be monitored quantitatively, reflecting the genotoxic potency. Bacteria such as *Escherichia coli* and *Salmonella typhimurium* respond to DNA damage via the SOS response [14]. This response leads to the coordinated induction of over 30 genes, which are under the control of the repressor LexA. This repressor binds specific sequence motifs (SOS boxes) present in the regulatory sequences of these genes. Genotoxic exposure leads to inactivation of LexA and consequently derepression of its regulon. The timing, as well as expression levels of the induced genes, is determined by the strength, number, and position of the SOS boxes in their promotor sequences. *sul*A (formerly *sfiA*), *umuDC*, *recN* and *recA* are among the most highly induced SOS genes [14]. The promoters of these genes/operons are of interest as these sequences could give the highest possible dynamic range in signal when fused to a reporter gene. These promoter sequences have been used in bacterial genotoxicity reporter assays such the SOS *umu* test (*umu* promoter), VITOTOX (*recN*) and the SOS Chromotest (*sulA*) [7, 8, 15, 16]. In particular, *sulA*, which in some studies was shown to

increase over 100-fold in its expression level when completely derepressed, was an obvious promoter to use in our reporter construct [17, 18]. While this work was in progress, Copp et al reported on a very effective application of a *sul*A::GFP reporter in *E. coli* [19]. This reporter was used to screen a library of nitroreductase variants generated by directed evolution techniques, to identify those most effective in bioactivating dinitrobenzamide prodrugs, by use of fluorescence activating cell sorting.

An increasing number of genes have been used in transcriptional fusions to monitor gene expression. The majority of genotoxicity reporters are based on fluorescence or bioluminescence, using fluorescent protein (FP) or luciferase fusion constructions [13]. Both reporters have specific advantages; however, FPs are particularly robust and do not need substrates or cofactors [13, 20]. We chose to use the green fluorescent protein (GFP) GFPmut3.1 [21]. In bacteria, this FP can be expressed to very high levels, has one of the shortest maturation times among FPs (5-7 min, important for real-time measurement), and has demonstrated a high quantum yield ("brightness") [22].

Chemicals tested for their genotoxicity also exert different levels of cytotoxicity and may interfere with the synthesis of the genotoxic reporter protein and thus its signal. A suitable correction for this effect can prevent underestimation of genotoxicity or even a false negative outcome and should be included in the test method [13]. Some bacterial reporter assays include optical measurements of cell growth (A_{600}) or the inclusion of an additional, constitutive expressing reporter strain or enzyme for this correction [13]. Optical density does not necessarily reflect cell viability. Moreover, bacterial cell division is halted upon genotoxic insult, inducing filamentation, which disrupts the correlation between optical density and bacterial cell number per volume [23]. Our approach includes an additional reporter strain for cytotoxicity testing using the same GFPmut3.1 reporter under the control of a synthetic constitutive promoter derived from the tetracycline

resistance gene of the transposon Tn10. Cytotoxicity measured by this approach will reflect the negative (cytotoxic) effects of the test compound and its dose on GFPmut3.1 expression, synthesis, and maturation.

Our laboratory has been involved in the development of bacterial cell systems that mimic human drug metabolism for in vitro genotoxicity assay applications. This work has sought to address the major limitations of in vitro mutagenicity tests by developing bacterial systems that contains human P450-mediated biotransformation capabilities and a genetic target for mutagenicity detection. These bacterial systems adequately reproduce the human P450 enzyme complex and have been used to study the role of P450-mediated pathways in chemical mutagenicity [24-29]. This article describes the development of several bacterial strains that include active human P450 biotransformation coupled with a genotoxicity and cytotoxicity reporter system for real-time measurements. Other laboratories have reported the use of human P450-containing genotoxicity reporter systems [30, 31]. However, these systems do not allow real-time measurements or effective high-throughput analysis due to the need for cell lysis and the addition of substrate for signal development.

Our system, containing a combination of a genotoxicity- and a cytotoxicity-reporter, was initially tested in four different bacterial backgrounds. Subsequently, the two systems that performed best were engineered to contain human P450 biotransformation expression constructs. We have focused on the incorporation of human CYP1A2, an important enzyme in the formation of reactive metabolites, as a proof of concept.

4.2. MATERIALS AND METHODS

4.2.1. Reagents

Ampicillin, kanamycin sulfate, chloramphenicol, isopropyl β-D-thiogalactoside (dioxane-free) (IPTG), thiamine, glucose, cumene hydroperoxide (CHP), 1-aminopyrene (1AP), 4-nitroquinoline-1-oxide (4NQO), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), *δ*-aminolevulinic acid (*δ*-Ala), and 2-aminoanthracene (2AA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Bacto agar, bacto peptone, bacto tryptone, bacto yeast extract, NB medium, and casamino acids were obtained from Difco (Detroit, MI, USA).

4.2.2. Cloning procedures

The regulatory sequence of the *E. coli sul*A gene was obtained by PCR amplification (primers Fw_sulA and Rv_sulA; Table S1), using genomic DNA isolated from *E. coli* K12 (strain C600) as template. The PCR product was cloned in the multicloning site of plasmid pCR2.1 (Invitrogen/Life Technologies, Grand Island, NY). Subsequently, the fragment containing the *sul*A promoter sequence was excised with *Sph*I and cloned into the *Sph*I site of plasmid pGFPmut3.1 (Clontech/Life Technologies; Mountain View, CA). After verification of the in-frame fusion of the *sul*A ATG start codon with that of GFPmut3.1 by sequencing, the *sul*A::GFPmut3.1 fragment was excised by digestion with *Not*I and *Sph*I and cloned into the human cytochrome P450 oxidoreductase (CPR) expressing plasmid pLCMhOR, thus creating phORSAG (Figure S1) [27]. Subsequently, the cDNA of human CPR was deleted by digestion with *Bam*HI and *Eco*O109I, the 3’ end was filled in with Klenow, and the DNA was ligated, resulting in plasmid pSAG (Figure S1). The 133 bp promoter-sequence of *sul*A including its SOS

box sequence matched NCBI Reference Sequence NC_000913.2, with the exception of two adjacent bases (CG) upstream of the translation start codon ATG due to the creation of the *Sph*I site. The constitutive GFPmut3.1 expressing plasmids were constructed using the minimal (constitutive) promoter sequences of the Tet resistance gene of Tn10 [32]. This promotor sequence was obtained by PCR amplification (primers Fw_Tet_*Xba*I and Rv_Tet_*Sph*I; Table S1) using genomic DNA of the *E. coli* K12 strain CA12184 containing *tol*C::Tn10 (*E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA). The PCR product was digested with *Sph*I and cloned in the phORSAG plasmid, previously treated with *Not*I, with the 3′ end filled in with Klenow and subsequently digested with *Sph*I, thereby substituting the promoter *sul*A sequence with that of the minimum TetR promoter sequence of Tn10, creating phORTc-gfp (Figure S1). Plasmid pTc-gfp was obtained by deletion of the CPR cDNA from phORTc-gfp by digestion with *Bam*HI and *Eco*O109I, the 3′end was filled in with Klenow, and the DNA was ligated. The minimal TetR promoter sequence (94 bp) was verified by sequencing (using primer Rv_GFP; Table S1) and demonstrated the correct in-frame fusion of the ATG start codon with that of the GFPmut3.1 gene. The 5'sequences of the TetR gene from Tn10 matched NCBI GenBank J01830.1 sequence, with the exception of two adjacent bases (CG) upstream of the translation start codon ATG due to the creation of the *Sph*I site. The mock plasmids pLCB and pLCBhOR (Figure S1) were obtained by *Bgl*I deletion or *Hpa*I deletion of the mutator *muc*AB operon of plasmid pLCM or plasmid pLCMhOR, respectively [27, 29]. Recently we found the CPR cDNA sequence in plasmids pLCBhOR, phORSAG and phORTc gfp, derived from pLCMhOR, to contain two deviations (resulting in amino acid substitutions Gly109Glu and Glu403Gly) from the consensus POR sequence NM_000941 [33]. These deviations were corrected by substitution of the *Bam*HI-*Eco*O109I fragment of plasmid pLCM_POR in these plasmids,

thus creating plasmids pLCB_POR, pSAG_POR, and pTc-gfp_POR, respectively [33]. Sequencing confirmed the consensus sequence of the CPR cDNA in these new plasmids. Cloning, propagation, and maintenance of plasmids were conducted using standard cloning *E. coli* strains. The heterologous expression of human CYP1A2 was obtained using the expression vector pCWh1A2 [34]. Its medium-high copy number parent plasmid pCW has a pMB1 replicon, which is compatible with the p15A replicon from the low copy number pLCM_POR derived plasmids (pLCB_POR, pSAG_POR and pTcGFP_POR) [35]. For transformation of the Ames tester strains TA1535 and TA100, plasmids were isolated and adapted to the restriction enzymes of *S. typhimurium* LT2 by passage through the restriction-deficient, methylation-proficient *S. typhimurium* strain LB5010 [36, 37]. Plasmids were transformed into tester strains using standard procedures.

4.2.3. Bacterial cultures and CPR/CYP1A2 expression

E. coli tester strains FP401 or PD301 and *S. typhimurium* strains TA1535 or TA100 containing either of the plasmids pSAG, pTcGFP or pLCB were cultured in LB medium (*E. coli*) or in NB medium (*S. typhimurium*), for 15 h at 37°C [25, 37, 38]. Media were supplemented with kanamycin (15 µg/mL, all strains), chloramphenicol (10 µg/mL, strain PD301), or ampicillin (25 µg/mL, strain TA100).

PD301 expressing CPR and CYP1A2 was cultured as described before [33]. Briefly, strains were cultured in Terrific Broth (TB) medium supplemented with peptone (2 g/L), thiamine (1 µg/mL), ampicillin (50 µg/mL), kanamycin (15 µg/mL), chloramphenicol (10 µg/mL), trace elements solution, (4 µL/mL), and 0.2 mM IPTG [39]. Cultures were started with 250 µL of –80°C glycerol stocks and were grown for 16 h at 28°C with moderate agitation.

Bacterial cultures for coexpression of CYP1A2 with CPR in TA1535 derived tester strains were performed as previously described by Fujita et al. with minor modifications [40]. Precultures were grown for 16 h at 37°C in Nutrient Broth medium supplemented with ampicillin (50 µg/mL) and kanamycin (15 µg/mL). Precultures were than diluted 1000-fold and grown for 8 h at 30°C in TB medium (pH 6.4) supplemented with peptone (2 g/L), thiamine (1 µg/mL), ampicillin (50 µg/mL), kanamycin (15 µg/mL), trace elements solution (4 µL/mL), and 0.1 mM *δ*-Ala [39]. Cultures were cooled on ice for 15-20 min, induced with IPTG (0.2 mM), and grown for an additional 18 h at 25°C with moderate agitation.

CYP1A2 expression levels, membrane preparations, and protein and CPR contents were obtained or determined as reported previously [33].

4.2.4. High-throughput genotoxicity assay

Bacterial cultures were concentrated by centrifugation, and the cell pellet was re-suspended in an equal volume of Vogel-Bonner medium E (VB medium), and the cell density (A_{600}) was determined [37]. Cultures were diluted in VB medium, containing glucose (0.5%) (w/v) and casamino acids (0.75 mg/mL), to a cell density of 0.050 A_{600}. Test compounds were diluted in VB medium and/or DMSO. Preliminary experiments were performed using a log dose-gradient of test compounds to obtain an effective dose range. The final gradients concentrations for each agent tested are as follows: 4NQO (0, 7.5, 15, 22.5, 30, 37.5, and 45 ng/well); MNNG (0, 60, 120, 180, 240, 300, 360, and 420 ng/well, except for derivatives of TA1535 and TA100, which were tested with 0, 25, 50, 75, 100, 125, 150, and 175 ng/well); CHP (0, 200, 400, 600, 800, 1000, 1200, and 1400 ng/well); 2AA (0, 2, 4, 6, 8, 10, 12, and 14 ng/well); IQ (0, 2, 4, 6, 8, 10, 12, and 14 ng/well) and 1AP (0, 100, 200, 300, 400, 500, 600, and 700 ng/well). When used, the

DMSO concentration was kept constant in all cases and was always ≤ 1 % (v/v). Test compound (10 µL) and diluted cultures (150 µL) were dispensed into the wells of flat bottom black 96-well microplates. Plates were covered with BREATHseal, placed in an orbital shaker, and incubated at 37°C with shaking. Fluorescence readings (λ_{ex}: 485 nm; λ_{em}: 510 nm) were performed at 90, 120, 150, 180, and 240 min in a microplate reader (Anthos Zenith 3100). Each assay contained two negative controls: one containing cell mixture and solvent and another containing solvent and diluted cells using VB medium without glucose or casamino acids supplementation. Positive controls using single dose of 4NQO (37.5 ng/well) were performed in all assays (with and without CYP1A2 expression), whereas positive controls using a single dose of 2AA (10 ng/well) were performed for strains with CYP1A2 expression. All dose levels were tested at least in triplicate.

4.3. RESULTS

For the development of prototypes of a bacterial high-throughput genotoxicity assay competent in human P450 biotransformation, we started by testing the principle of the assay by constructing strains for the detection of compounds that directly cause DNA damage.

4.3.1. Bacterial strains

The bacterial backgrounds used for development of our detection systems contained specific characteristics that enhance their sensitivity to detect potential mutagens (Table S2). *S. typhimurium* strains TA1535 and TA100 were derived for use in the Ames assay [37]. Both strains carry mutations in the *uvr*B gene, which enhances the SOS response, and in *rfa*, which increases the permeability of the cell envelope. In

addition, TA100 carries a mutator plasmid, pKM101. *E. coli* tester strains FP401 and PD301 were developed in our laboratory. Both have mutations in *rfa* as well as in *uvr*A, which also enhance the SOS response. Furthermore, strain PD301 has mutations in the *ada* and *ogt* genes, which lead to reduced repair of DNA alkylation. Our previous work demonstrated these strains are suitable for the coexpresssion of human P450 and its reductase [25, 27, 41]. Each of the four bacterial systems was transformed with the SOS-inducible GFP reporter plasmid, the constitutive GFP reporter plasmid, or a control plasmid lacking the *gfp* gene, enabling correction for any fluorescence generated by media/cell constituents or the test compound and/or metabolites (Table S2). Thus, for each of the four bacterial hosts, three tester strains were created: the inducible genotoxicity measuring strain (e.g. TA1535-I), the cytotoxicity measuring strain (e.g. TA1535-C), and the blank strain (e.g. TA1535-B).

4.3.2. GFP fluorescence dose-response plots, induction factors, and SOS inducing potency

For each dose level, the GFP fluorescence of the I-strains and their corresponding C-strains (for genotoxicity and cytotoxicity, respectively) was obtained and corrected for background fluorescence (obtained with the B-strains.. Subsequently, GFP fluorescence dose-response curves were plotted for the I-strains and their corresponding C-strains (Figure 1).

The GFP fluorescence obtained for both the I-strains and C-strains were corrected for their GFP fluorescence at the start of incubation (t = 0 min) to correctly compare the test among the four different bacterial backgrounds at the start of the incubation. The ratio of GFP fluorescence of the I-strain divided by the fluorescence obtained for the C-strain

for dose level *x* appropriately reflects the induction of the *sulA* reporter for that particular dose level. As such, the Induction Factor (F_I) at each dose level was calculated by:

$$F_I = \frac{f_I^x / f_I^0}{f_C^x / f_C^0}$$

in which f_I^x, f_I^0, f_C^x, f_C^0 represent the GFP fluorescence measured at *x* and zero dose levels for the corresponding I- and C-strains, respectively. F_I can be plotted against each of the tested dose levels. The SOS-Inducing Potency (SOSIP) can be derived from the linear part of this curve (using ANOVA with a 95% confidence interval) in the same manner as that calculated with the SOS Chromotest [16]. SOSIP represents the molar potency of each compound at inducing *sul*A-driven GFP expression.

4.3.3. Assay condition optimization

Assays were performed in a 96-well microplate format. Plates were incubated at 37°C, and several assay conditions were verified for optimal outcomes. These assay conditions included incubation volumes, cell density at starting point, aeration (shaking speed), and the top reading *z*-height (height of the reading sensor in the microplate reader) (data not shown). Optimal conditions were fixed, and assays were performed with an incubation volume of 160 µl (150 µL cells + 10 µL test compound), a starting cell density of A_{600}=0.050, a shaking speed of 200 rpm, and a *z*-height of 0.3 mm. Fluorescence readings were performed at 0, 90, 120, 150, 180, and 240 min incubation times. The 96-well format permitted the testing of a single compound in triplicate at eight different dose levels, for the I-, C-, and B- strains on one plate, including controls. Alternatively, this format permitted the testing of a compound at 12 different dose levels in duplicate for the I-, C-, and B-strains. This last format was used in all preliminary experiments using log-dose gradients of test compounds. When a compound tested positive for one or more of

the log doses, then an appropriate linear dose gradient was determined for accurate SOSIP determinations.

4.3.4. High-throughput genotoxicity/DNA damage assay using four tester bacterial strains with direct DNA damaging compounds

E. coli and *S. typhimurium* prototype tester bacteria sets were tested with three well-known direct acting mutagens, 4NQO, MNNG, and CHP. The GFP fluorescence dose-response curves for 4NQO are depicted in Figure 1 (for GFP fluorescence dose-response curves for MMNG and CHP, see Figures S3 and S4, respectively). F_I plots of all three compounds are presented in Figure 2.

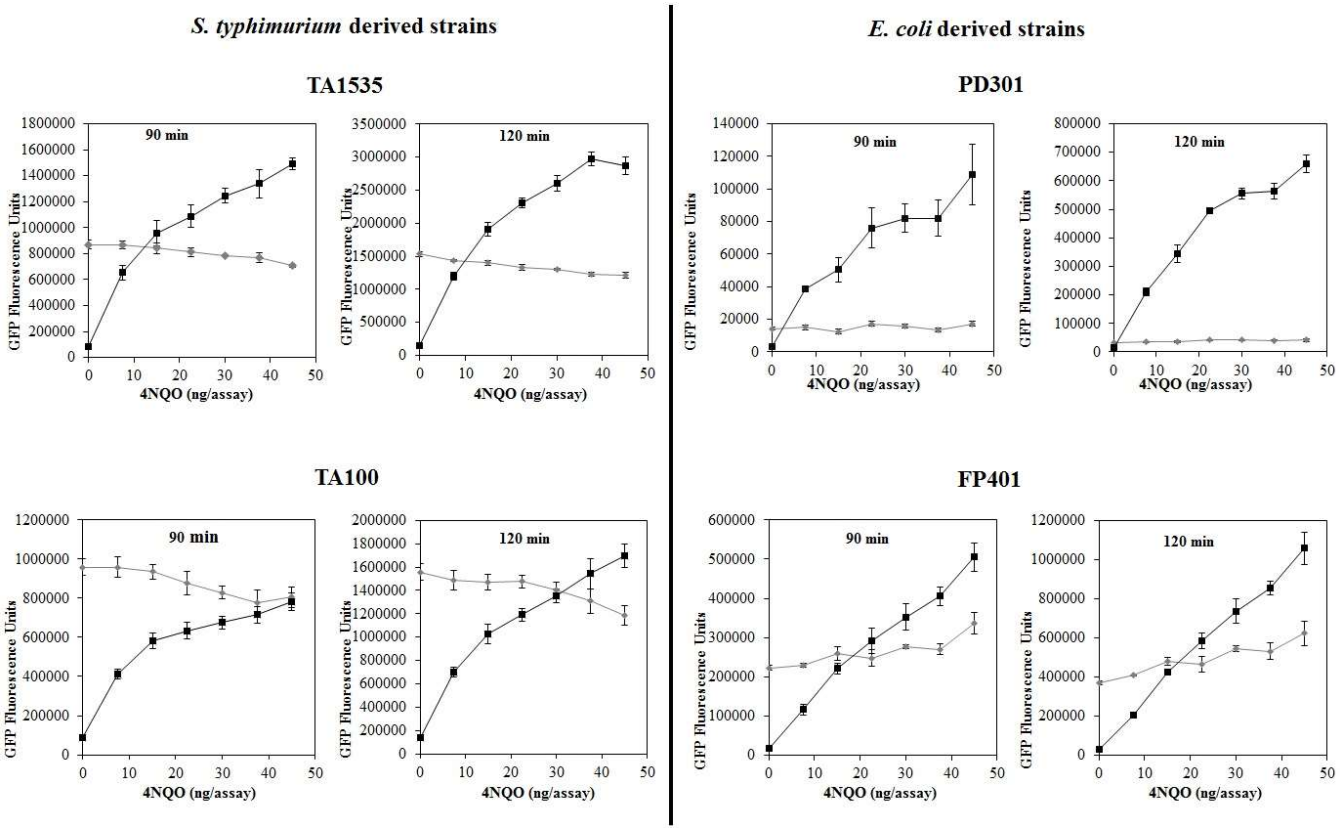

Figure 1. GFP fluorescence dose-responses curves of 4NQO for the I- and C-strains derived from *S. typhimurium* strains TA1535 and TA100 and *E. coli* strains FP401 and PD301 (for simplicity, only the curves for 90 and 120 min incubation times are presented; black lines: pSAG (I-strains); gray lines: pTcGFP (C-strains)).

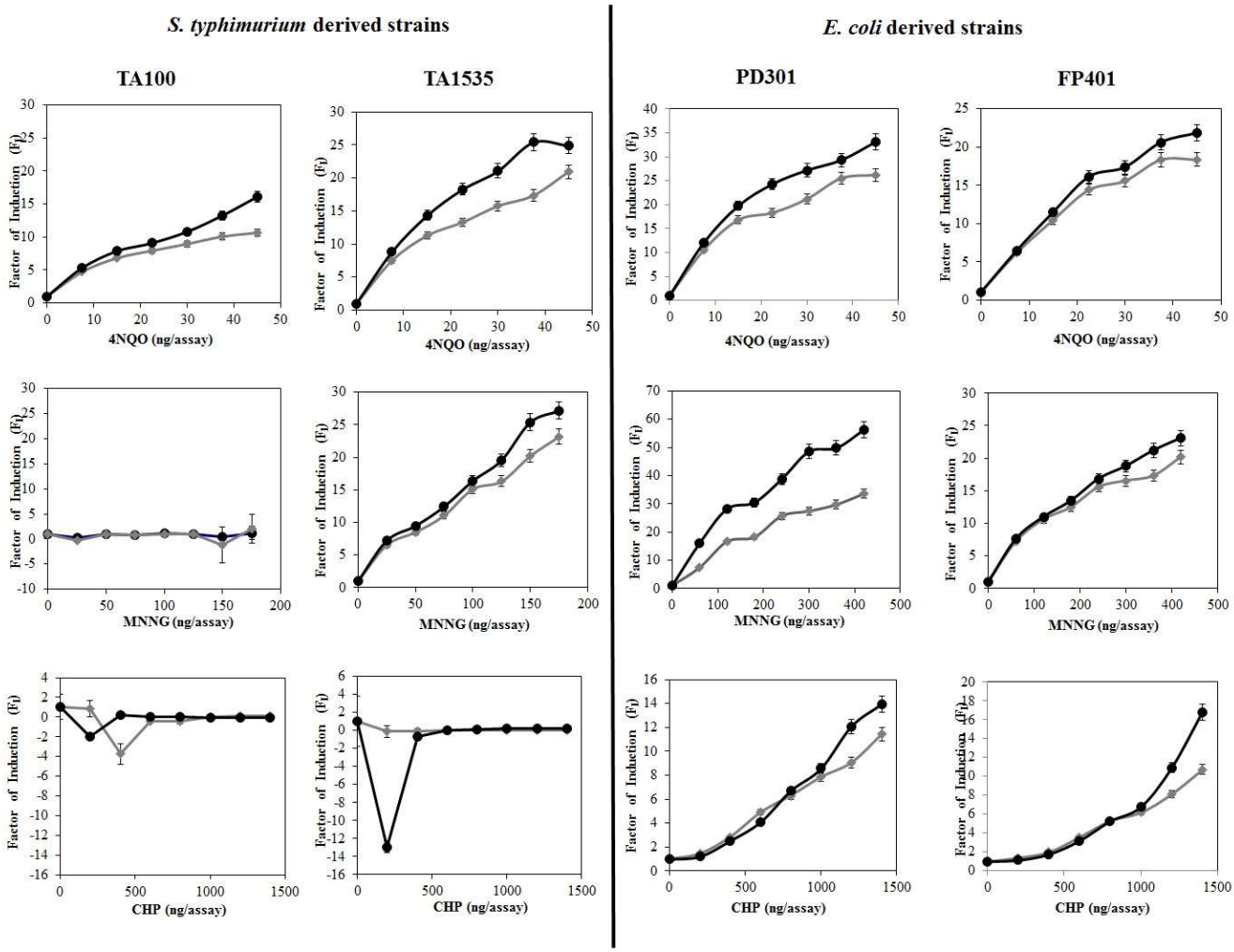

Figure 2. Induction Factor (F_I) plots of 4NQO, MNNG, and CHP obtained with the *S. typhimurium* systems derived from strains TA1535 and TA100 as well as with *E. coli* systems derived from strains FP401 and PD301 (for simplicity, only the plots for 90 and 120 min incubation times are presented). No dose-response was detected with the TA100-derived system for MNNG and CHP. No dose-response was detected with the TA1535-derived system for CHP (gray lines: 90 min; black lines: 120 min).

4NQO tested positive with the four prototypes using the different bacterial backgrounds and the same dose gradient. In all cases, maximum responses were obtained at 120 min (Table 1). The *S. typhimurium* and *E. coli* derived set of strains demonstrated different sensitivities toward MNNG, resulting in the use of different dose gradients. The TA100-derived system did not show any activity for this DNA alkylating mutagen up to

a maximum dose level of 1 μg/well (data not shown). The other three prototypes were positive for MNNG, reaching maximum F_I values after 120 min of incubation (Figure 2).

CHP did not demonstrate a positive response in the TA1535- or the TA100-derived systems with doses up to 10 μg/well (data not shown). However, this oxidative mutagen demonstrated a positive response in *E. coli* backgrounds FP401 and PD301, reaching a maximum F_I after 120 min of incubation.

The SOS induction potency of 4NQO, MNNG, and CHP were calculated for each of the four prototype systems and are summarized in Table 1.

Table 1. SOSIP values of 4NQO, MNNG and CHP Using Four Different Bacterial Backgrounds

[a]Values are per nanomole of test compound and represent mean ± SD; N ≥ 3. ---: no SOSIP could be derived.

	SOSIP[(a)]					
bacterial	**4NQO**		**MNNG**		**CHP**	
background	**90 min**	**120 min**	**90 min**	**120 min**	**90 min**	**120 min**
TA1535	76 ± 8	118 ± 10	18 ± 1	22 ± 1	---	---
TA100	43 ± 6	58 ± 4	---	---	---	---
FP401	113 ± 5	128 ± 3	6 ± 1	7 ± 1	0.9 ± 0.1	1.7 ± 0.3
PD301	113 ± 16	164 ± 24	15 ± 2	22 ± 2	1.2 ± 0.1	1.7 ± 0.1

4.3.5. Human P450 competent high-throughput genotoxicity assay prototypes

On the basis of the results of the assay with direct acting DNA-damaging agents, the two most effective strains, TA1535 and PD301, were used in the development of human P450-competent systems for genotoxicity assays. As a proof of concept, we created heterologous systems containing human CYP1A2 with CPR using our previously developed biplasmid system for their coexpression in bacteria [25, 35]. This system allows for the efficient expression of these human proteins and their correct insertion into the bacterial cell membrane, with physiologically relevant CPR/P450 stoichiometries and

with enzyme activities that replicate the enzymatic activity found in vivo [42]. This approach has been in use for over a decade in our laboratory and has given consistent CPR/P450 stoichiometries in bacterial cell models [25, 26, 33, 41, 43].

4.3.5.1. CYP1A2 and CPR expression

Before use in the assay, cultures were always characterized for their P450 expression levels. The I-, C- and B-strains all produced similar levels of the P450 holoenzyme when grown under the same conditions on the same day. Daily values ranged between from 214 to 251 nmol/L (Figure S2). To verify if the consistency in CPR/P450 ratio was maintained, the CPR and P450 expression levels were determined in membrane preparations of the different strains. Contents ranged from 8.5 to 12.1 pmol/mg for CPR and 79.9 to 90.1 pmol/mg for CYP1A2, confirming the consistency of CPR/P450 ratio (see Table S3).

4.3.5.2. Human CYP1A2-competent genotoxicity prototype test systems for the detection of DNA-reactive metabolites

Three carcinogens, IQ, 2AA, and 1AP, were assayed with the two human CYP1A2-expressing systems. All three compounds generated robust positive responses with the two CYP1A2-expressing systems (Figure 3 and Table 2; for GFP fluorescence plots of the I- and C-strains, see Figures S5 and S6). No response was obtained when testing the same dose gradient of these three compounds with the two bacterial systems devoid of human CYP1A2 enzyme (data not shown).

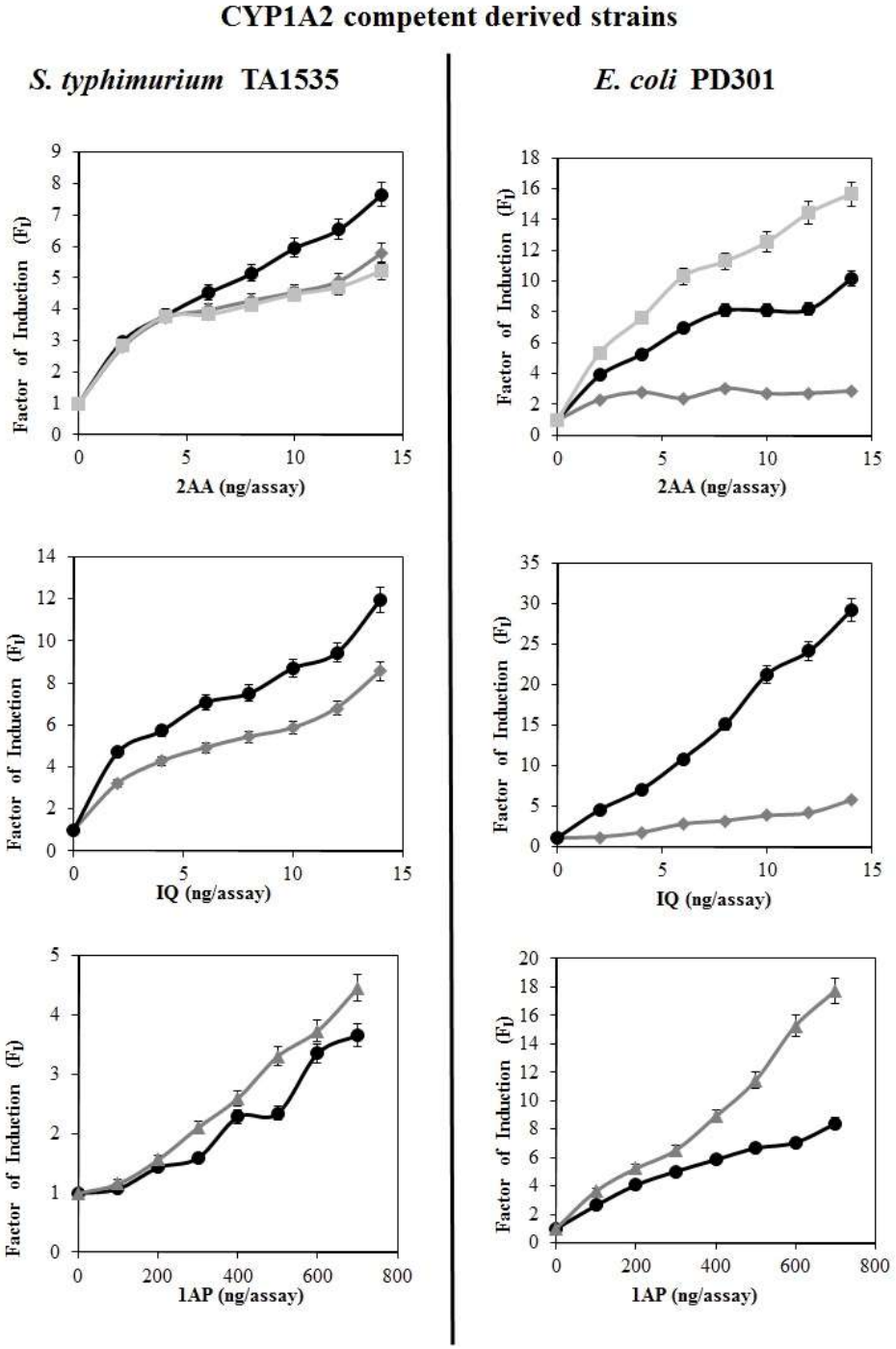

Figure 3. Induction factor (F_I) plots of 2AA, IQ, and 1-AP using human CYP1A2-competent TA1535- and PD301-based assays (diamonds: 90 min; circles: 120 min; squares: 150 min; triangles: 180 min).

Table 2. SOSIP values of 2AA, IQ, and 1AP using two human CYP1A2-Competent Bacterial Backgrounds.

	SOSIP[(a)]						
bacterial	**2AA**			**IQ**		**1AP**	
background	**90 min**	**120 min**	**150 min**	**90 min**	**120 min**	**120 min**	**180 min**
TA1535_1A2	54 ± 8	82 ± 6	47 ± 9	90 ± 9	130 ± 14	0.9 ± 0.1	1.1 ± 0.1
PD301_1A2	28 ± 12	110 ± 15	189 ± 17	65 ± 5	404 ± 17	2.1 ± 0.1	5.1 ± 0.3

[a]Values are per nanomole of test compound and represent mean ± SD; N ≥ 3.

Both TA1535- and PD301-based bacterial systems competent in CYP1A2 demonstrated maximum SOSIP values at 120 minutes for IQ and 180 min for 1AP. In the case of 2AA, the maximum SOSIP was reached at 120 min when using the CYP1A2-competent TA1535 bacterial background, versus 150 min when using the CYP1A2-competent PD301 background.

4.4. DISCUSSION

Reactive metabolites are known to be involved in adverse drug reactions. For 50% of current drugs, metabolism by P450 is the major clearance pathway and is a key determinant in performance and safety [12, 44]. Experimental in vitro data is important to confirm in silico analysis and to minimize the likelihood that important structural (in silico) alerts will be overlooked during the chemical design phase. This is of increasing importance as newer and more complex drug structures are introduced that take full advantage of the breadth of chemical space available for pharmacological targets. Identification of competing pathways, and catalysis by polymorphic enzymes, including individual differences in human biotransformation, add complexity to the identification of potential metabolite formation as well as ranking of compounds [44, 45].

The current in vitro high-throughput genotoxicity testing methods are limited in detecting relevant reactive metabolites due to inadequate representation and/or failure to incorporate a human biotransformation system. This issue is a general limitation of in vitro toxicity test systems for in vitro to in vivo extrapolations [46].

In this article, we described the development of bacterial real-time detection systems to detect DNA damage that are highly sensitive, require short assay times, and are competent in human cytochromes P450. These systems maximize the dynamic range of the signal by using one of the most highly inducible genes that reacts to DNA damage, namely, *sul*A. The reporter GFPmut3.1, a GFP variant with one of the shortest maturation times and with a high quantum yield, was chosen to minimize assay duration and enable effective real-time measurements. The assay also includes a reporter to correct for possible cytotoxic effects of the test compound, making use of the same GFP as the genotoxicity reporter. A different FP could have been used for this purpose (with different excitation/emission properties to distinguish between the two signals), which would enable the use of a single bacterial strain for both the genotoxicity and cytotoxicity reporters; however, FPs differ considerably in maturation times [22]. These differences would interfere with the interpretation of the genotoxicity signal.

Initially, we tested four different bacterial backgrounds, namely, TA1535 and TA100, two *S. typhimurium* strains used in the Ames test, and strains FP401 and PD301, two *E. coli* strains developed in our laboratory, that were used for the construction of human P450-competent mutagenicity tester bacteria. The prototypes were tested with three direct-acting mutagens. When responding, all four prototypes demonstrated a maximum response (SOSIP) after 120 min of exposure. The usefulness of including a cytotoxicity reporter strain (C-strains) in the assay is best demonstrated by the results obtained with CHP, a compound with a high level of toxicity (Figure S4).

The TA1535-based assay was unable to detect the oxidative mutagen CHP, as was the case of the TA100-based system. These two *S. typhimurum* strains, part of the original set of tester strains developed by the Ames laboratory, are less appropriate for the detection of oxidative mutagens, which triggered the development of tester strain TA102 for this purpose [47]. Interestingly, strain TA100 (the bacterial system of the VITOTOX test, containing a DNA damage (*rec*N)- inducible reporter) is equivalent to TA1535 but also contains the mutator plasmid pKM101 [37]. These types of plasmids are used in bacterial mutagenicity tests to increase mutation frequencies after genotoxic exposure which results from mutagenic translesion DNA transcription (mediated by the plasmid-borne *muc*AB genes); however, this causes a concomitant downregulation of the SOS response [35, 48]. Although it is favorable for bacterial mutagenicity assays, this type of mutator plasmid might not be adequate for use in SOS-based reporter assays, and our preliminary results indicated this is the case. When comparing the two *E. coli* derived systems, the PD301 background demonstrated increased sensitivity for detecting the genotoxicity of 4NQO, MNNG, and CHP relative to that of the FP401-derived system. This finding corroborates our previous study in which strain PD301 was demonstrated to be more sensitive in the detection of chemical mutagenicity when compared to strain FP401 due to the inactivation of the *ada* and *ogt* genes present in PD301 [25]. The functionality of our reporter assay was demonstrated from the results obtained with 4NQO and MNNG. These direct-acting mutagens gave SOSIP values of 71 and 0.9 per nmol, respectively, in the SOS Chromotest, a test also based on a *sul*A reporter fusion [49]. When tested with the TA1535- and the PD301- derived systems, 4NQO gave SOSIPs of 118 and 163 per nmol, respectively, whereas MNNG an SOSIP of 22 per nmol for both strains. Although it was tested with only a low number of direct-acting DNA-damaging compounds, the principle of the assay was demonstrated by using the TA1535-

and the PD301-derived systems. Responses were found in very short assay times (120 min), with relatively low (nanogram range) dose levels.

Subsequently, the two most effective bacterial systems, based on strains TA1535 and PD301, were adapted to contain human P450 biotransformation capabilities using the biplasmid system for coexpression of CYP1A2 and its redox partner CPR, along with the GFP reporters [25, 28]. The low level of inducer (0.2 mM IPTG) that we use for coexpression of P450 and CPR with the biplasmid expression system leads to very reproducible expression levels of both of these human proteins [25, 41, 43]. Moreover, we have demonstrated that this approach leads to CYP1A2 activities that are comparable with those in human liver, in alkoxyresorufin dealkylation reactions, and in the metabolism of two therapeutic drugs (clozapine and phenacetine) [27, 41]. Within individual experiments, the I-, C-, and B-strains demonstrated equivalent expression levels of CYP1A2 and CPR in each of the two systems (Table S3). These results imply equivalent CYP1A2 biotransformation capabilities among the genotoxicity and cytotoxicity reporter strains as well as the mock strains used. Differences in CYP1A2 biotransformation capabilities between the tester strains of the same bacterial background (TA1535- or PD301 –derived) would have caused incorrect F_I and SOSIP results. Furthermore, the similar CYP1A2 and CPR expression levels in both the *E. coli* and *S. typhimurium* derived systems allowed for a direct comparison of these two types of reporter assays to the bacterial background used and not to differences in P450 biotransformation capacities. The CPR/P450 ratios for the different strains were very similar (between 0.10 and 0.13), which are well within in the range observed in human liver microsomes (0.08–0.5) (see Table S3) [50, 51].

2AA, IQ and 1AP are well-known to form DNA-reactive metabolites through CYP1A2-mediated metabolism [52]. Genotoxicity of these three compounds was readily

detected using dose levels in the nanogram range. Remarkably, maximum SOSIP values were obtained at different incubation times. Both systems gave maximum SOSIP values after 120 min for IQ and 180 min for 1AP. However, 2AA reached maximum SOSIP values at 120 min when using the TA1535-derived system and 180 min for the PD301 system. This may be explained by differences in bacterial N- and O-acetyl-transferase capacities between the PD301- and TA1535- derived systems. In addition to CYP1A2-mediated oxidation, heterocyclic and aromatic amines require a subsequent N-acetyl or O-acetyl conjugation to generate DNA-damaging metabolites [53]. *S. typhimurum* tester strains of the Ames assay have been shown to contain very efficient bacterial N- and O-acetyl transferases [54]. Since both bacterial systems contain equivalent CYP1A2 activities, the more efficient acetylation capability of the TA1535-derived system of CYP1A2-generated 2AA metabolites may be responsible for reaching earlier maximum SOSIP levels.

Although it was tested with a very limited number of compounds, the two bacterial CYP1A2 systems presented here have the appropriate characteristics for the detection of DNA-reactive metabolites in a high-throughput manner. These characteristics include a short assay time (120-180 min), real-time measurement, the use of small amounts of test compound (less than a few micrograms), and adaptability to a microplate format. To our knowledge, the only genotoxicity test using human P450 containing bacterial strains is based on the *umu* test, an end-point assay requiring cell lysis and the addition of substrate. This procedure is incompatible with real-time measurements and, unlike the assay presented here, is unsuitable for true high-throughput screening [30, 31].

The systems described here will require further validation for specificity and reproducibility and for confirmation of their high-throughput characteristics. This validation can be performed using additional known CYP1A2 substrates that produce

genotoxic and nongenotoxic metabolites. These bacterial systems may be further optimized using bacterial backgrounds other than the four presented here. Additional improvements could be made through the use of different reporter fusions, both in the promoter sequence used (i.e., the SOS promoter) or in the reporter gene. the specific characteristics of these assays should be maintained in future developments of enhanced versions, in particular the dynamic range (promoter induction levels) and very short signal development time (for real time measurements). Further refinements could include the integration of reporter genes or cDNAs encoding human biotransformation enzymes into the bacterial chromosome, creating more stable and robust systems and allowing the creation of a battery of specific human biotransformation competent high-throughput genotoxicity test assays.

Human CYP1A2 has been used here to provide a proof of concept, but it can be exchanged for other human P450 isoforms, as the major human drug metabolizing P450s are readily expressed in bacteria [35]. Detoxification of reactive metabolites by other human metabolic systems is not represented in our prototype assays and thus they may be prone to overestimate reactive metabolite formation. However, it is important to characterize the capacity of human P450 in generating DNA-damaging metabolites of a compound, especially in the case of saturated or diminished capacity of detoxifying routes, i.e., by drug-drug interactions or genetic polymorphism of conjugating enzymes.

Concerns are often raised regarding the relevance of models based on bacterial cells versus models based on mammalian or human cell lines. However, the Ames assay, developed more than 3 decades ago, is still part of standard in vitro genotoxicity assays and continues to demonstrate the usefulness of bacterial-based assays. Our bacterial systems were developed to provide a method that would more accurately predict which compounds could produce DNA-reactive metabolites in humans. Moreover, the short cell

cycle of bacteria (relative to those of mammalian cells) allows a fast turnaround in data output. In addition, bacterial systems have low costs and ease of use in these applications. Both of these properties are important for screening a large number of drug candidates to detect their chemical liabilities. Recently, the application of a multistrain (bacterial) bioreporter bioassay for drug screening was reported, including reporters for DNA damage, heat shock, and oxidative stress [55]. However, no human drug metabolism capability was incorporated into this system; thus, it provides only information regarding the parent compounds, not their metabolites.

In summary, we describe here the development of bacterial test systems for real-time detection of DNA-reactive metabolites. The systems are based on bacterial strains that produce active human P450, namely, CYP1A2, and lend themselves to adaptation to a high-throughput assay format. These attributes include the ability to be run in microplate format, requiring only a small amount of test chemical, having a short turnaround time for data output, and producing real-time measurements. These systems are prototypes that can be expanded to include additional human biotransformation capabilities.

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4.5. SUPPORTING INFORMATION

Table S1. PCR primers

Fw_sulA	AAGATTAATTTATGTTTTCCCGTCACCA
Rv_sulA	TAA*GCATGCT*CAATCCAGCCCCTGTGAGTTACTGTA (*SphI*)
Rv_GFP	CCATTAACATCACCATC
Fw_Tet_XbaI	CTAA*TCTAGA*CATCATTAATTCCT (*XbaI*)
Rv_Tet_SphI	GAACTAT*GCATGC*CACTTTCTCTAC (*SphI*)

Table S2. Strains

Strains	Description	Reference
S. typhimurium		
TA1535	*hisG46, gal, Δ(chl, uvrB, bio), rfa*	[1]
TA100	TA1535, pKM101	[1]
E. coli		
FP400	AB1157, *uvrA6, galE, his*$^{+}$*, pro*$^{+}$*, leu*$^{+}$*, thr*$^{+}$	[2]
FP401	FP400*, rfa*	[2]
PD300	FP400, ada^{-} [Tet^{s}], *Δ* ogt::cm^{r}	[3]
PD301	PD300, *rfa*	[3]

Table S3. P450 and CPR contents of membrane preparation

		CYP (pmol/mg protein) *	CPR (pmol/mg protein) *	CPR/P450
***S. typhimurium* TA1535**	**B**	79.9 ± 0.1	9.2 ± 0.2	0.12
	C	83.2 ± 0.1	8.8 ± 0.2	0.11
	I	81.0 ± 0.1	8.5 ± 0.2	0.10
***E. coli* PD301**	**B**	85.2 ± 0.1	10.9 ± 0.3	0.13
	C	90.1 ± 0.1	12.1 ± 0.4	0.13
	I	87.1 ± 0.1	11.4 ± 0.4	0.13
Human liver microssomes				0.2 [0.08-0.5] [a]

* Values represent mean ± Standard Deviation, N=3.

[a] CPR content in human liver microsomes from Venkatakrishnan et al. [4], and total P450 content in human liver microsomes from Paine et al [5].

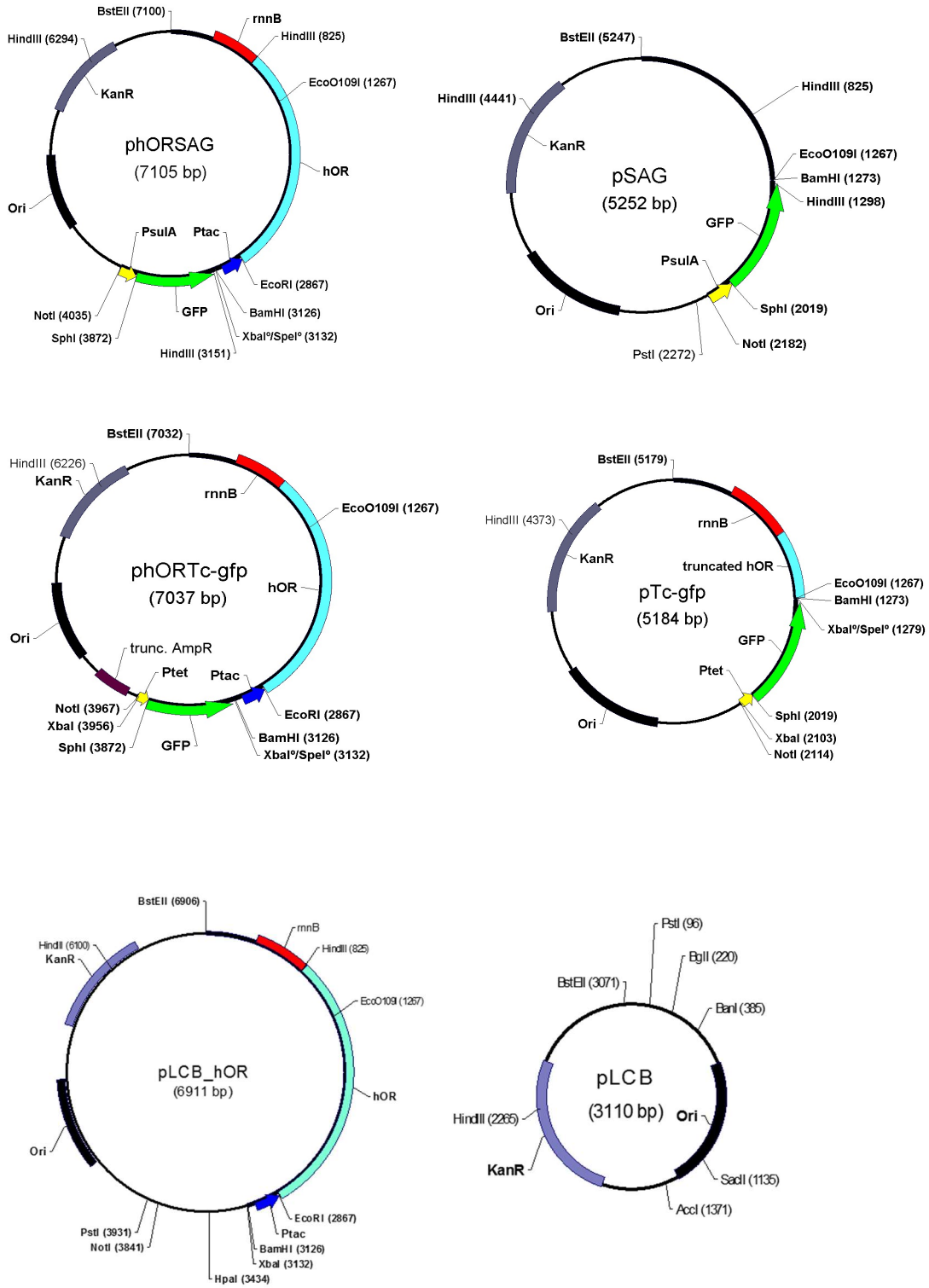

Figure S1. Plasmids used in the I-, C-, and B- strains.

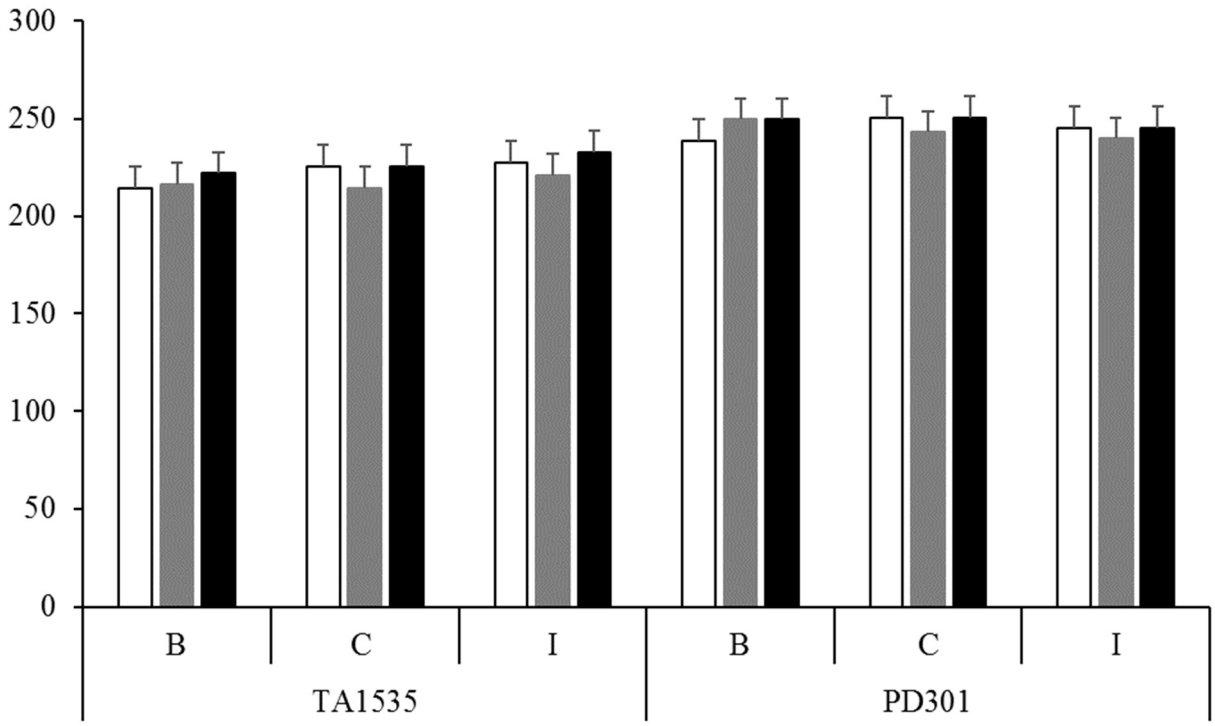

Figure S2: P450 contents determined in whole cells of the different strains cultured on three different days (white, gray and black bars represent experimental day 1, 2 and 3, respectively).

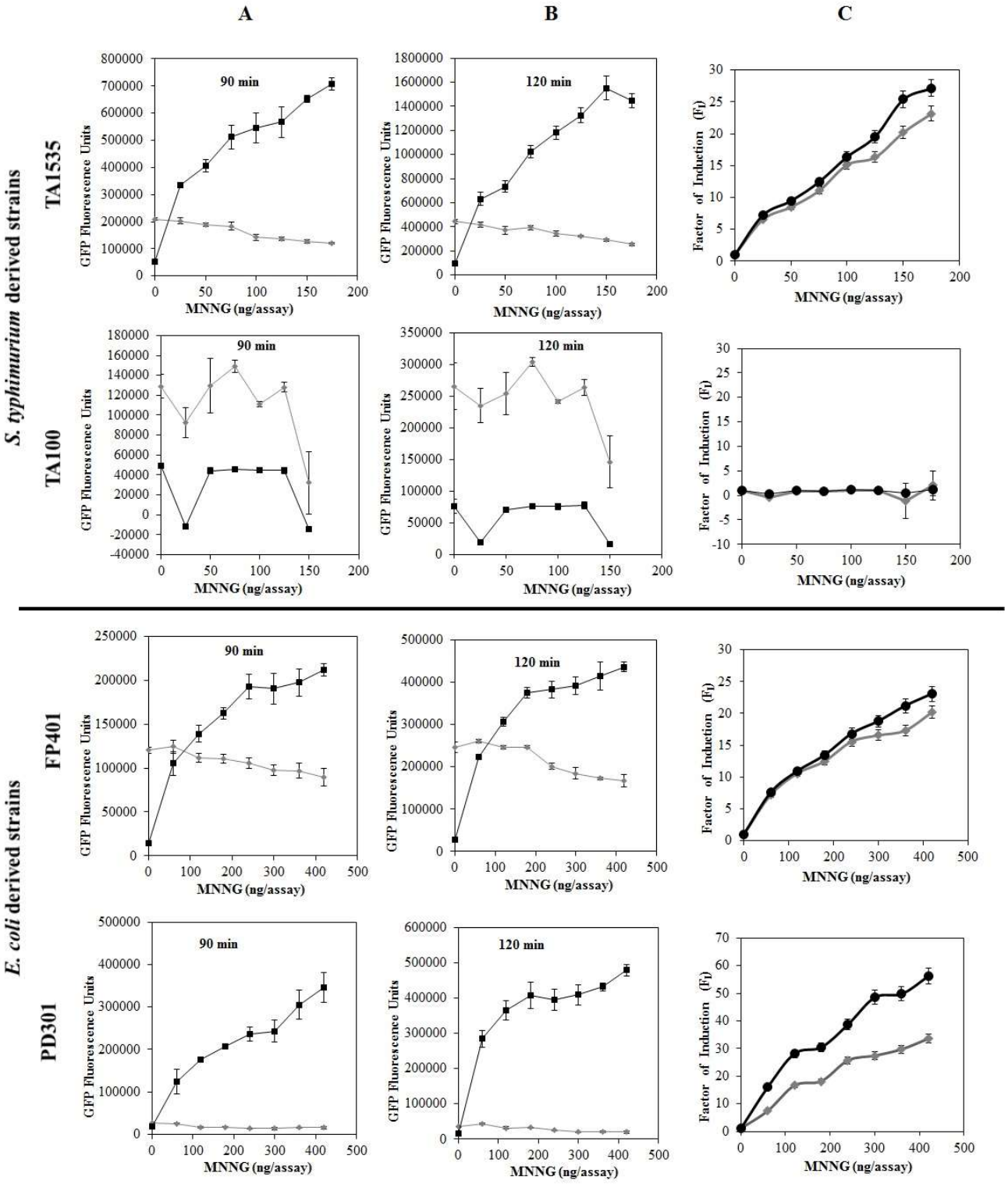

Figure S3. A) and **B)** GFP fluorescence dose-response curves of MNNG after 90 and 120 min incubation, respectively (black lines: pSAG (I-strains); gray lines: pTcGFP (C-strains); **C)** F_I plots of MNNG. (diamonds: 90 min; circles: 120 min incubation).

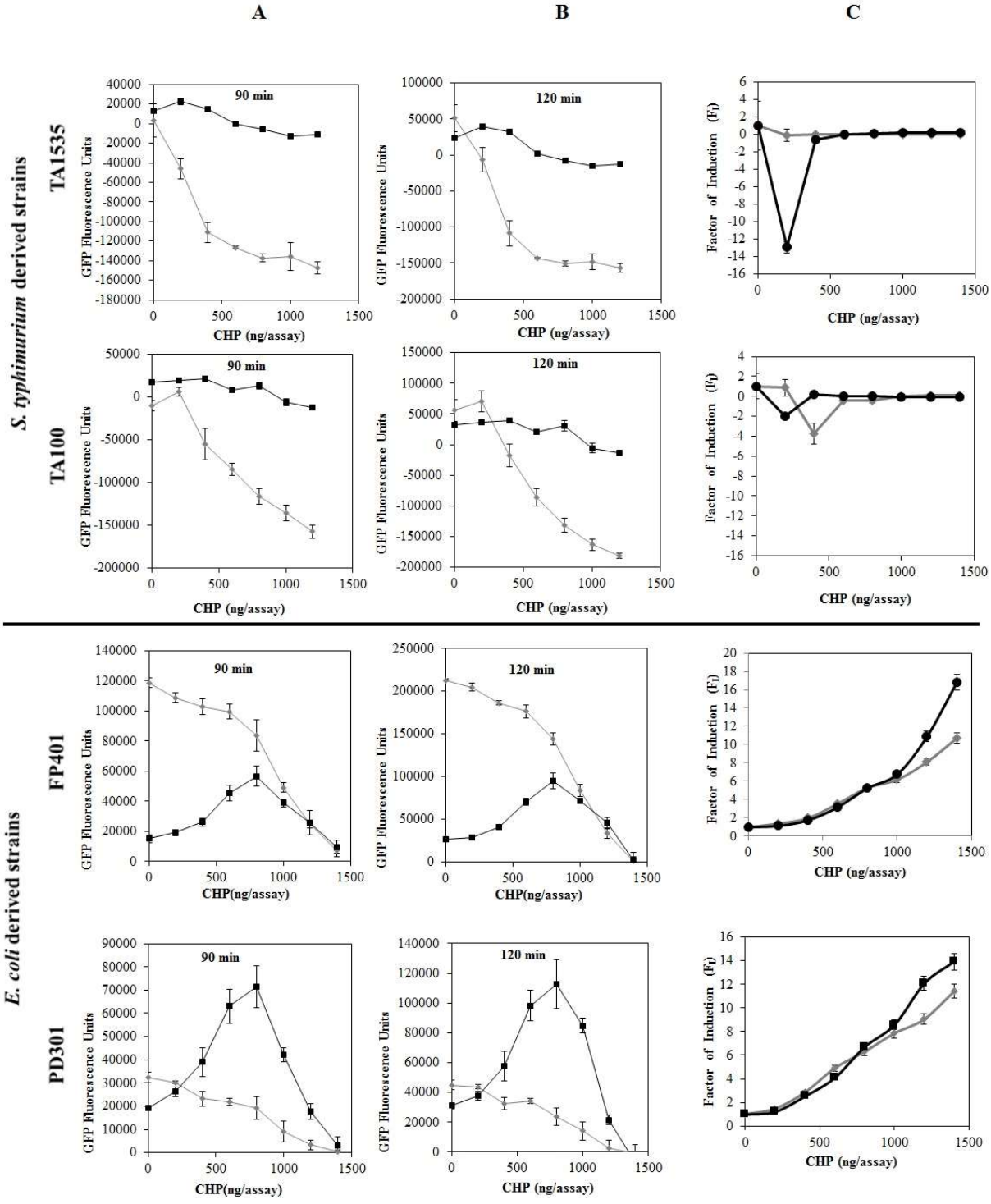

Figure S4. A) and **B)** GFP fluorescence dose-response curves of CHP after 90 and 120 min incubation, respectively (black lines: pSAG (I-strains); gray lines: pTcGFP (C-strains); **C)** F_I plots of CHP (diamonds: 90 min; circles: 120 min incubation).

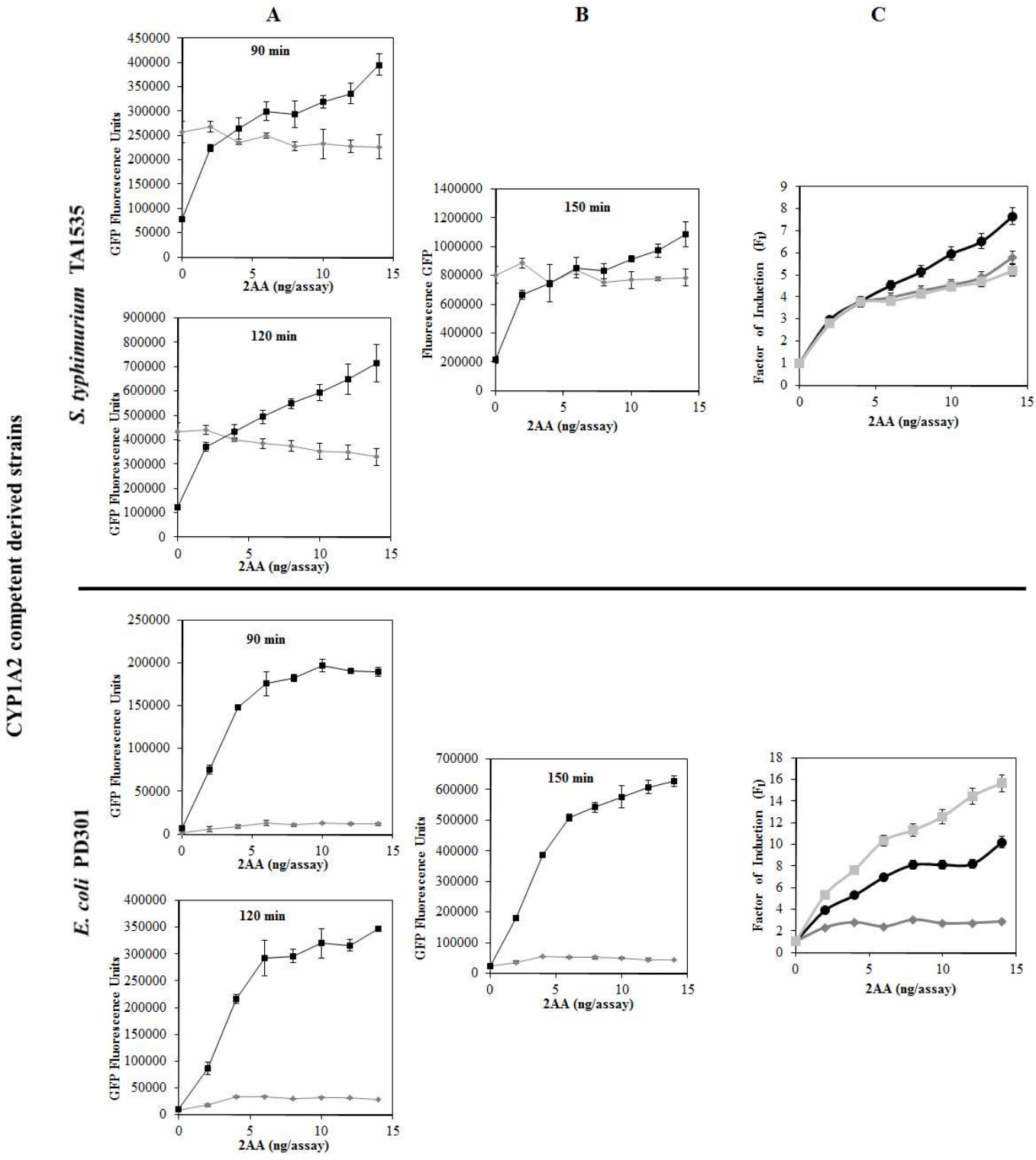

Figure S5. **A)** and **B)** GFP fluorescence dose-response curves of 2AA after 90 and 120 min incubation (black lines: pSAG (I-strains); gray lines: pTcGFP (C-strains); **C)** F_I plots of 2AA (diamonds: 90 min; circles: 120 min; squares 150 min incubation).

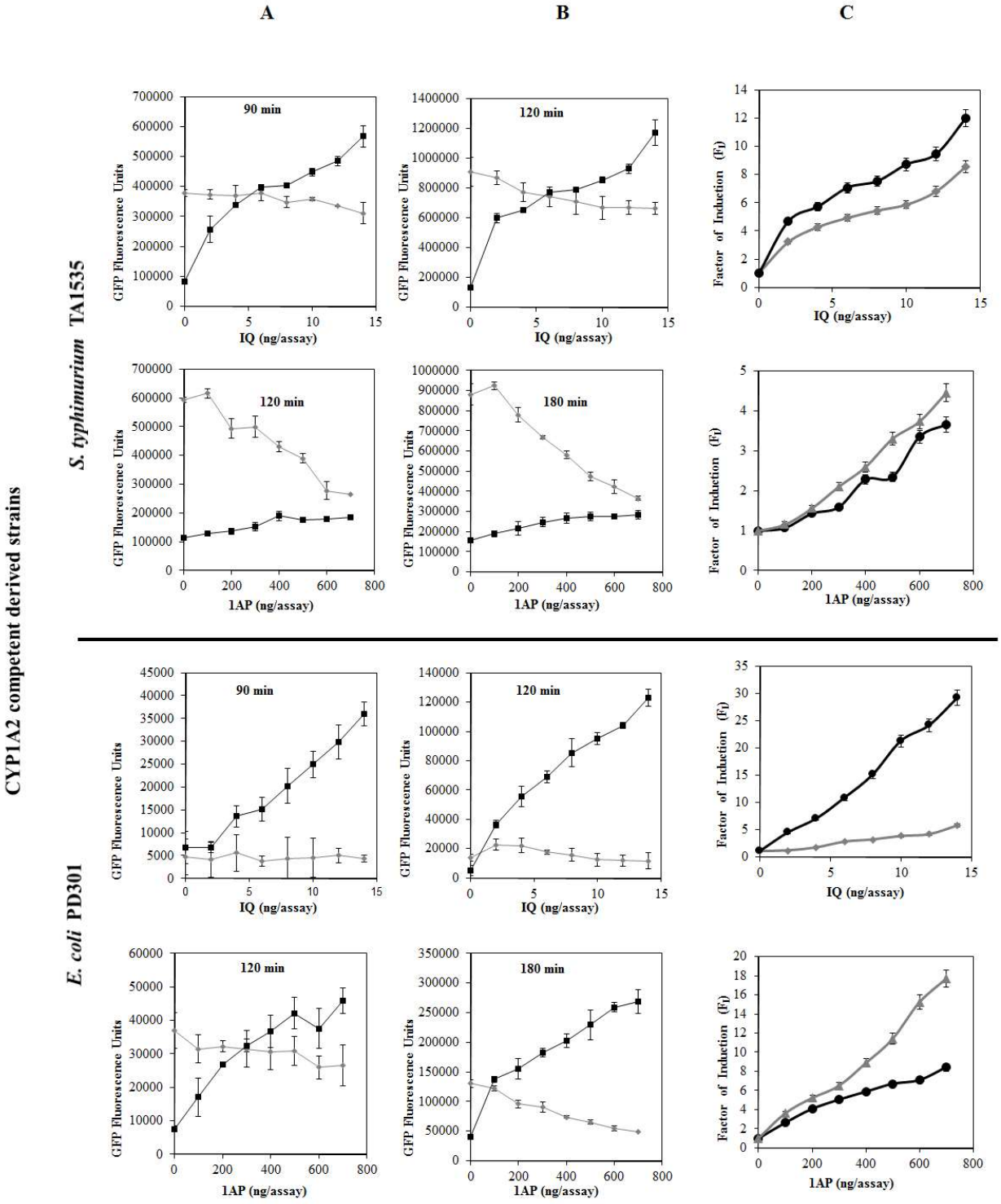

Figure S6. A) and **B)** GFP fluorescence dose-response curves of IQ and 1AP after 90 and 120 min incubation, respectively (black lines: pSAG (I-strains); gray lines: pTcGFP (C-strains); **C)** F_I plots of IQ and 1AP (diamonds: 90 min; circles: 120 min; triangles: 180 min incubation).

4.5.1. References

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4. Venkatakrishnan, K., et al., *Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches.* Drug Metab Dispos, 2000. **28**(12): p. 1493-504.
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CHAPTER 5

Cytochrome P450 expression system for high-throughput real-time detection of genotoxicity: Application to the study of human CYP1A2 variants

Adapted from: Palma, B.B.; Moutinho, M.; Urban, P.; Rueff, J.; Kranendonk, M.; *"Cytochrome P450 expression system for high-throughput real-time detection of genotoxicity: Application to the study of human CYP1A2 variants"* Mutat Res Genet Toxicol Environ Mutagen. 2016 Aug; 806:24-33.

ABSTRACT

Individual variations in cytochrome P450-mediated metabolism are believed to contribute to individual susceptibility to chemical carcinogenesis. CYP1A2 is one of the major forms of cytochrome P450 involved in drug metabolism and bioactivation of carcinogens. We have applied a recently developed high-throughput *Salmonella typhimurium* TA1535 system for detection of DNA damaging agents to the study of CYP1A2 polymorphisms. Non-synonymous variants T83M [CYP1A2*9], S212C [CYP1A2*12], S298R [part of CYP1A2*21], G299S [CYP1A2*13], I314V [no allele designation], I386F [CYP1A2*4], C406Y [CYP1A2*5] and R456H [CYP1A2*8] were examined. The cDNAs for each of these variants and the wild-type were co-expressed with human NADPH cytochrome P450 oxidoreductase in the TA1535-based system. The bioactivation capacity of these CYP1A2 variants was investigated using three CYP1A2-dependent pro-mutagens, 1-aminopyrene (1AP), 2-aminoanthracene (2AA), and 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ). All CYP1A2 variants except R456H, T83M, and I386F gave positive responses with all three compounds. Variant R456H generated no detectable holoenzyme and no detectable response for any of the compounds; I386F did not bioactivate IQ; T83M did not bioactivate 1AP. Multivariate analysis indicated variant T83M to be substantially altered in catalytic properties when compared with wild-type CYP1A2; variants G299S and I386F are slightly but significantly different. These results corroborate our previous studies, indicating the effectiveness of this new high-throughput system, not only for examining the effect of CYP1A2 polymorphisms on pro-mutagen bioactivation, but also for obtaining insights on CYP1A2 function at the mechanistic level.

5.1. INTRODUCTION

The time and cost required to perform in vivo testing hamper the early stages in drug development [1]. Moreover, animal toxicity testing has shown poor concordance between predicted and actual adverse drug reactions (ADRs) in humans [2]. Sensitive and efficient methods for evaluation of the propensity of drug candidates to form reactive metabolites (RMs) are needed. High-throughput (HT) screening assays are appropriate for handling the flow of new drug candidates in a systematic and time-efficient manner [3]. However, ADMET profiling does not address the influence of human genetic polymorphisms [4]. The extensive inter-individual variability in xenobiotic and drug metabolism presents a major clinical problem [5]. Even at the same dose, drug plasma levels can vary more than 1000-fold between individuals [6]. Such variations can arise from genetic polymorphisms of drug-metabolizing enzymes (DMEs) and also from physiological, patho-physiological, and environmental factors [7]. Genetic factors are considered to account for 20-40% of the differences between individuals, in both the therapeutic response and the toxic effects of drugs and xenobiotics [8].

Polymorphisms of DMEs are strongly implicated in inter-individual differences in overall exposures to both the parent drug and RMs, affecting drug response and the risk of ADRs [9]. Cytochrome P450s (CYPs) are responsible for the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds [10]. CYP is the enzyme system most frequently involved in drug metabolism, along with two additional major enzyme classes, esterases and UDP-glucuronosyl transferases [11, 12]. The majority of CYP-mediated xenobiotic metabolism is carried out by highly polymorphic forms [13] and represent a key factor in inter-individual differences in drug response [14]. Furthermore, several ADRs and/or lack of efficacy of therapeutic drugs can be explained by the presence of CYP polymorphisms [15, 16]. Approximately 40%

of the 157 different pharmacogenomic drug labels listed by FDA are associated with variations in CYP genes [17]. Two recent studies identified over 6000 single nucleotide variations scattered across the 57 human CYP genes, 50% being novel and more than 90% being rare [14, 18]. Verification of the phenotypic consequences of these large numbers of CYP variants will require highly efficient screening systems for in vitro toxicology and genotoxicology studies.

CYP families 1- 3 are responsible for 70-80% of the metabolism of clinically used drugs [19] and are involved in the biotransformation of a large number of xenobiotics. The human CYP1A family consists of CYP1A1 and CYP1A2. CYP1A1 is expressed primarily in extra-hepatic tissues, while CYP1A2 is almost exclusively expressed in the liver, representing about 4-16% of total CYP content of that organ [20]. Inter-individual differences in CYP1A2 activity are well known; variations of up to 60-fold have been reported [21, 22]. In addition, CYP1A2 expression is highly inducible: variations of approximately 15-fold in mRNA content and 40-fold in protein levels have been observed in human liver [23, 24]. To date, 41 CYP1A2 haplotypes have been designated by the Human Cytochrome P450 Allele Nomenclature Committee [25].

Over the past two decades, our group has established several bacterial cell systems competent in recombinant human CYP biotransformation [26-28]. This work has sought to address the major limitations of in vitro mutagenicity tests by developing bacterial systems that express human CYP-mediated biotransformation capabilities and a genetic target for mutagenicity detection. These systems have been used for the study of eight non-synonymous polymorphic variants of human CYP1A2 [29, 30]. We have recently reported on the development of bacterial HT-test systems containing active human CYP1A2, for the rapid, real-time detection of DNA reactive metabolites [31].

The primary objective of this study was to evaluate the effectiveness of these new HT systems for the study of polymorphic genetic variants of CYP1A2, using the wild type (WT) and eight CYP1A2 variants: T83M [CYP1A2*9], S212C [CYP1A2*12], S298R [part of CYP1A2*21], G299S [CYP1A2*13], I314V [no allele designation], I386F [CYP1A2*4], C406Y [CYP1A2*5] and R456H [CYP1A2*8] (Supplemental Fig. 1). As a proof of concept, three pro-mutagens, 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ); 2-aminoanthracene (2AA), and 1-aminopyrene (1AP) (Fig. 1), were tested to detect the presence of CYP1A2-dependent genotoxicity. The data was evaluated through multivariate statistical analysis and interpreted using the CYP1A2 crystal structure [32].

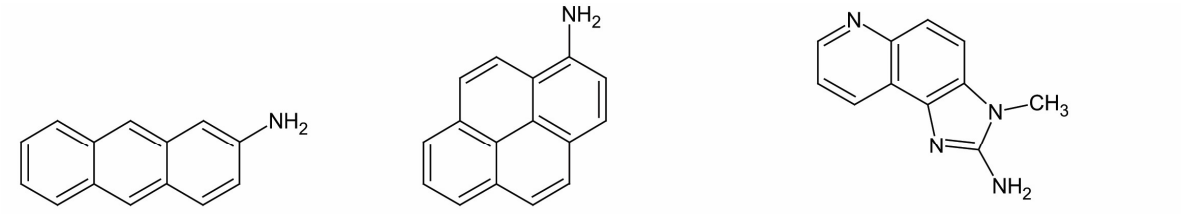

Figure 1. Chemical structures of the three CYP1A2 dependent pre-mutagens used in this study.

5.2. MATERIALS AND METHODS

5.2.1. Reagents

Ampicillin, kanamycin sulfate, chloramphenicol, isopropyl β-D-thiogalactopyranoside (dioxane-free) (IPTG), thiamine, glucose, 1-aminopyrene (1AP), and 2-aminoanthracene (2AA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ) was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Bacto agar, bacto peptone, bacto tryptone, bacto yeast extract, NB-medium, and casamino acids were obtained from Difco (Detroit, MI, USA).

5.2.2. Bacterial tester strains

The recently developed bacterial HT-test systems expressing active recombinant human CYP1A2, for the rapid detection of DNA reactive metabolites [31], are based on the bacterial strains *S. typhimurium* TA1535 [33] or *E. coli* PD301 [26]. The detection method relies on a fusion between the promoter sequence of *sul*A (one of the most highly inducible genes that responds to DNA damage) with a reporter, GFPmut3.1, GFP variant with one of the shortest maturation times and high quantum yield. This allows a short assay duration and effective real-time measurements. The assay includes a general cytotoxicity measurement by which possible effects on formation of the genotoxicity reporter signal can be corrected [31]. Due to its real-time measurement capability, the DNA damage potency (SOS inducing potency or SOSIP) can be derived at different incubation times during one single assay.

Strain TA1535 was transfected with the expression vector encoding WT human CYP1A2 wildtype (WT) or one of eight CYP1A2 variants (Supplemental Fig. 1) [29]. Each of these was combined with one of the three human cytochrome P450 oxidoreductase (CPR) expression/reporter vectors (pLCB_POR, pTcGFP_POR, or pSAG_POR) (Table 1) [31]. Transfections were performed by standard electroporation procedures.

Table 1. Strains used in this study.

Strains	Description	Reference
TA1535	*hisG46, gal, Δ(chl, uvrB, bio), rfa*	[33]
TA15235/CYP1A2_I	TA1535/pCWh1A2/pSAG_POR	[31]
TA15235/CYP1A2_C	TA1535/pCWh1A2/pTc-gfp_POR	[31]
TA15235/CYP1A2_B	TA1535/pCWh1A2/pLCB_POR	[31]

Plasmids	Description	Reference
pCWh1A2	*E. coli* exp. vector, pCWori^{+} derived, Amp^{r}, ptac.ptac/LacI^{q}, containing modified cDNA of human CYP1A2 WT or CYP1A2 variant;	[29, 51]
pSAG_POR	derivative of pLCMhOR, Δ*mucAB,* Kan^{r}, containing cDNA of human CPR under tac promotor, containing cDNA of GFPmut 3.1 under *sul*A promotor.	[31]
pTc-gfp_POR	derivative of pLCMhOR, Δ*mucAB,* Kan^{r}, containing cDNA of human CPR under tac promotor, containing cDNA of GFPmut 3.1 under synthetic *Tet*R promotor.	[31]
pLCB_POR	derivative of pLCMhOR, Δ*mucAB,* Kan^{r}, containing cDNA of human CPR under tac promotor.	[31]

5.2.3. Bacterial cultures and CYP1A2/CPR expression

Bacterial growth with co-expression of the CYP1A2 WT and variants with human CPR, membrane preparations, and determinations of protein content (Bradford assay) were performed as reported previously [27, 30, 31, 34]. The CYP contents of whole cells and membrane preparations were determined using CO-difference spectrophotometry, while the CPR content of membrane preparations was determined using the NADPH-cytochrome c reduction assay, as described previously [27, 30, 31, 34].

5.2.4. High-throughput genotoxicity assay

The assay was performed as described previously [31]. Briefly, bacterial cultures of the TA1535 derived I-, C-, and B-strains (Table 1) were diluted with VB medium [33] containing glucose (0.5%, w/v) and casamino acids (0.75 mg/mL) to A_{600} = 0.05. Test compounds were diluted in VB medium and/or in DMSO. The volume of DMSO used

was kept constant in all assay wells and was ≤1% of the total assay volume. Final pre-mutagen doses were as follows: 2AA and IQ: 0, 2, 4, 6, 8, 10, 12, and 14 ng/well; 1AP: 0, 100, 200, 300, 400, 500, 600, and 700 ng/well. Test compound (10 µL) and diluted cultures (150 µL) were dispensed into the wells and the microplates were covered with BREATHseal™. Each assay contained two negative controls: cell mixture plus solvent; and solvent plus diluted cells, using VB medium without glucose/ casamino acids supplementation. The covered microplates were placed in an orbital shaker and incubated at 37 °C with 200 rpm shaking. Fluorescence readings (λ_{ex}: 485 nm; λ_{em}: 510 nm) were performed at 90, 120, 150, and 180 min using a microplate reader (Anthos Zenith 3100). All doses were tested at least in triplicate. GFP-specific fluorescence was obtained by subtracting background fluorescence obtained with the B-strain. Subsequent calculations of the Induction Factors (F_I) and SOS Inducing Potencies (SOSIP) were performed as described before [31]. Briefly, the F_I at each dose level was calculated by:

$$F_I = \frac{f_I^x / f_I^0}{f_C^x / f_C^0}$$

in which $f_I^x, f_I^0, f_C^x, f_C^0$ represent the GFP fluorescence measured at x and zero dose level, for the corresponding I- and C-strains, respectively. The Induction Factors can be plotted against each of the tested doses. The SOSIP was derived from the linear part of this curve (using ANOVA with 95% confidence interval). SOSIP represents the molar potency of each compound in inducing *sul*A-driven GFP expression.

5.2.5. Statistics

The data set used for multivariate statistical analysis consisted of the SOSIP values for the four measured time points, for each of the three tested compounds. The normalization procedure was based on the variance in the dataset. Both the column and row variances were applied as described previously [35]. The CYP1A2 activities of the three different pre-mutagens were analyzed using principal component analysis (PCA) and multidimensional scaling (MDS). PCA visualizes systematic patterns or trends of variation in large data sets. The trends of variation hidden in the initial multidimensional space are made evident by constructing new orthogonal axes in the projected space (the two first principal components), derived from the directions of greatest variability [36]. MDS is a nonlinear projection of the distances separating each object from the others in the original multidimensional space into a 2- or 3-dimensional diagram designated as the MDS configuration plot [37]. MDS enables the visualization and evaluation of the distances between two variants, considering the influence of all other variants at the same time. Kruskal's stress, a diagnostic index which varies from 0 (a perfect fitting) up to 1, (no fitting) was used as to evaluate the quality of MDS analysis; it measures the closeness of the distance mapping in the 2D MDS plot compared to the "real" distances in the original space. The multivariate statistics and dendogram constructions (Pearson correlation and MDS dendogram) were performed and obtained by using Addinsoft XLSTAT software.

5.3. RESULTS

5.3.1. CYP1A2 and CPR expression

Before use in the assay, cultures were characterized for their CYP expression levels, determined in whole cells. The set of the I-, C- and B-strains (Table 1) containing

one of the nine forms of CYP1A2 (Supplemental Fig. 1) produced the same level of P450 holoenzyme, when grown under the same conditions on the same day (Fig. 2). Daily expression levels of CYP in whole cells ranged from 117 to 251 nmol/l for all forms, with the exception of the I386F and R456H variants. The CYP holoenzyme level for the I386F variant was significantly lower (approximately one-fifth) than that found with the CYP1A2 WT, while the R456H variant did not show any detectable holoprotein. To verify consistency of CPR/CYP stoichiometries, CPR and CYP levels were determined in membrane preparations for each CYP1A2-containing strain (see Table 2). Contents ranged from 8.3-13.4 pmol/mg for CPR and 72.5-85.2 pmol/mg for CYP, except for membranes of the I386F and R456H variants, which did not contain detectable CYP holoenzyme. CPR/CYP ratios ranged from 0.10-0.13.

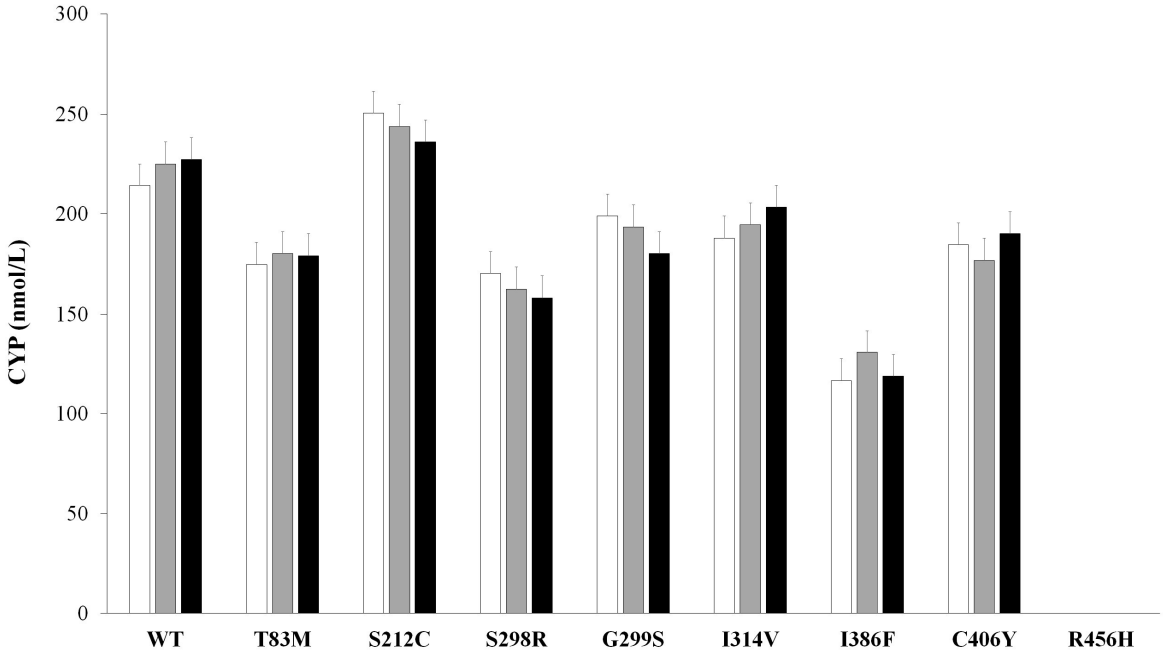

Figure 2. Expression levels (nmol/L) of CYP1A2 wildtype and its variants expressed in the B- (white bars), C- (gray bars) and I- strains (black bars), determined in whole cells.

Table 2. CYP and CPR contents in membrane preparations and respective ratios.

CYP1A2 variant [a]	CYP (pmol/mg protein) [b]	CPR (pmol/mg protein) [b]	CPR/CYP
WT	79.9 ± 0.1	9.2 ± 0.2	0.12
T83M	76.7 ± 0.1	9.6 ± 0.2	0.13
S212C	85.2 ± 0.1	8.3 ± 0.2	0.10
S298R	76.8 ± 0.1	10.3 ± 0.4	0.13
G299S	87.0 ± 0.1	11.4 ± 0.3	0.13
I314V	72.5 ± 0.1	9.7 ± 0.3	0.13
I386F	--	12.5 ± 0.3	
C406Y	82.4± 0.1	10.5 ± 0.3	0.13
R456H	--	13.4 ± 0.4	
Human liver microsomes			0.2 [0.08-0.5] [c]

[a] Values for each CYP1A2 variant are representative when expressed either in the blanc (B), cytotoxicity measuring (C) or inducible genotoxicity measuring (I) strains.

[b] Values represent mean ± standard deviation, N=3; --: undetectable.

[c] CPR content in human liver microsomes from Venkatakrishnan et al. [52], and total CYP content in human liver microsomes from Paine et al. [53].

5.3.2. HT genotoxicity testing with CYP1A2 variants

Three CYP1A2 dependent promutagens, 1AP, 2AA, and IQ, (Fig.1) were assayed for genotoxicity with the TA1535-derived system, expressing human CYP1A2 WT and the eight CYP1A2 variants (see Fig. 3A and 3B for F_I plots). The SOSIP values are presented in Fig. 4A-C, and Supplemental Table 1A-C. The three compounds showed no genotoxicity in the HT assay in reporter strains that did not express the CYP1A2 system (data not shown). Variant R456H did not generate a positive response with any of the compounds (see Fig. 3B).

A

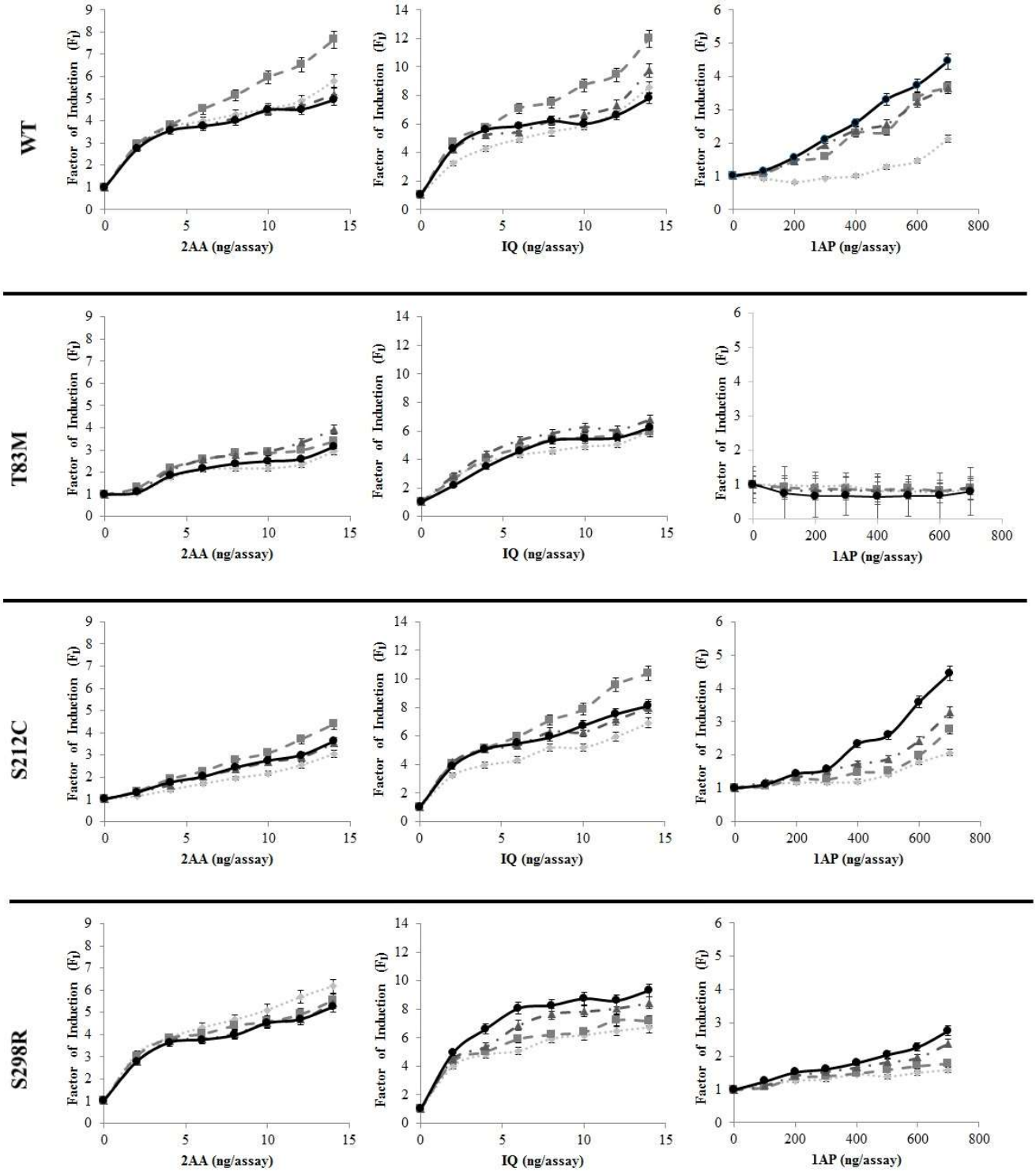

Figure 3. F_I plots for the TA1535 derived HT system, containing the different CYP1A2 variants and WT for 2AA, IQ and 1AP at different incubation times (light gray/dotted lines/diamonds: 90 min; medium gray/trace lines/squares: 120 min; dark gray/dote-trace lines/triangles: 150 min; black/continuous lines/circles: 180 min).

B

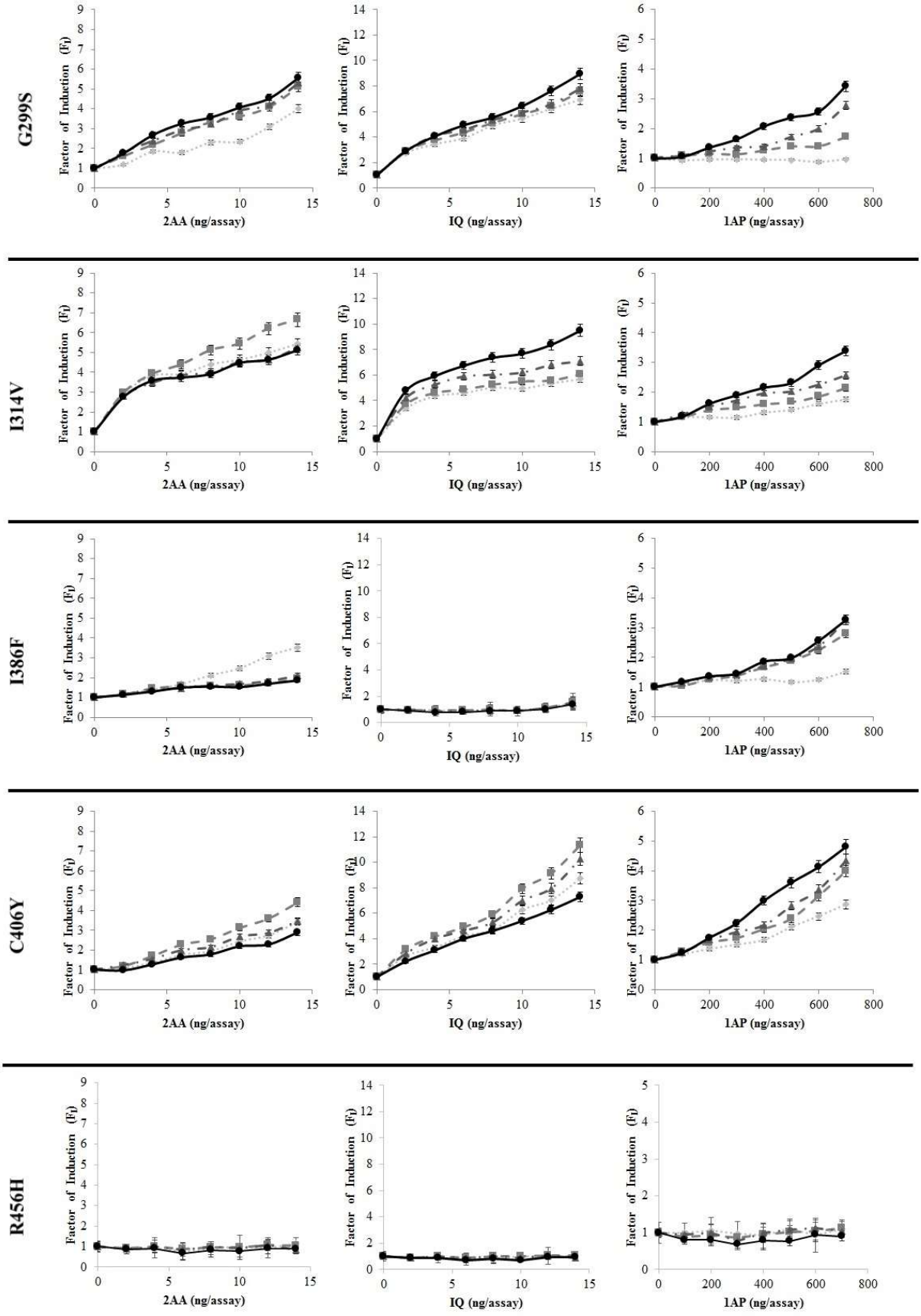

Figure 3. (*Continued*).

When compared to CYP1A2 WT, most of the CYP1A2 variants demonstrated a slight decrease in the maximum SOSIP for 2AA, except for variants I314V and S298R, which demonstrated CYP1A2 WT-like values (see Fig. 4A and Supplemental Table 1A). Maximum SOSIP values were reached after 150 and 180 min incubation time for variants T83M and G299S, respectively, while the S298R and I386F variants reached their maximum values at 90 min. The remaining variants reached their maximum response at 120 min, as was found for CYP1A2 WT.

All variants demonstrated similar maximum SOSIP values for IQ when compared to CYP1A2 WT, except for variants I386F and R456H. The I386F and R456H variants showed no genotoxicity (see Fig. 4B and Supplemental Table 1B), even up to 240 min incubation (data not shown). Variants S298R, G299S, and I314V reached their maximum F_I's and consequently maximum SOSIP values after 180 min incubation, whereas variants S212C and C406Y behaved as the CYP1A2 WT, reaching maximum SOSIP values at 120 min. Variant T83M showed maximum SOSIP after 150 min.

The responses for 1AP were the most discriminating among variants (see Fig. 4C and Supplemental Table 1C). Variants S212C and C406Y showed similar SOSIP maximums compared to the CYP1A2 WT. However, all other variants demonstrated reduced capacity to bioactivate this promutagen. Interestingly, no response could be detected for variant T83M, while G299S began to show activity only after 120 min of incubation, indicating less efficient 1AP bioactivation capacity. All other variants reached their maximum SOSIP values at 180 min incubation time.

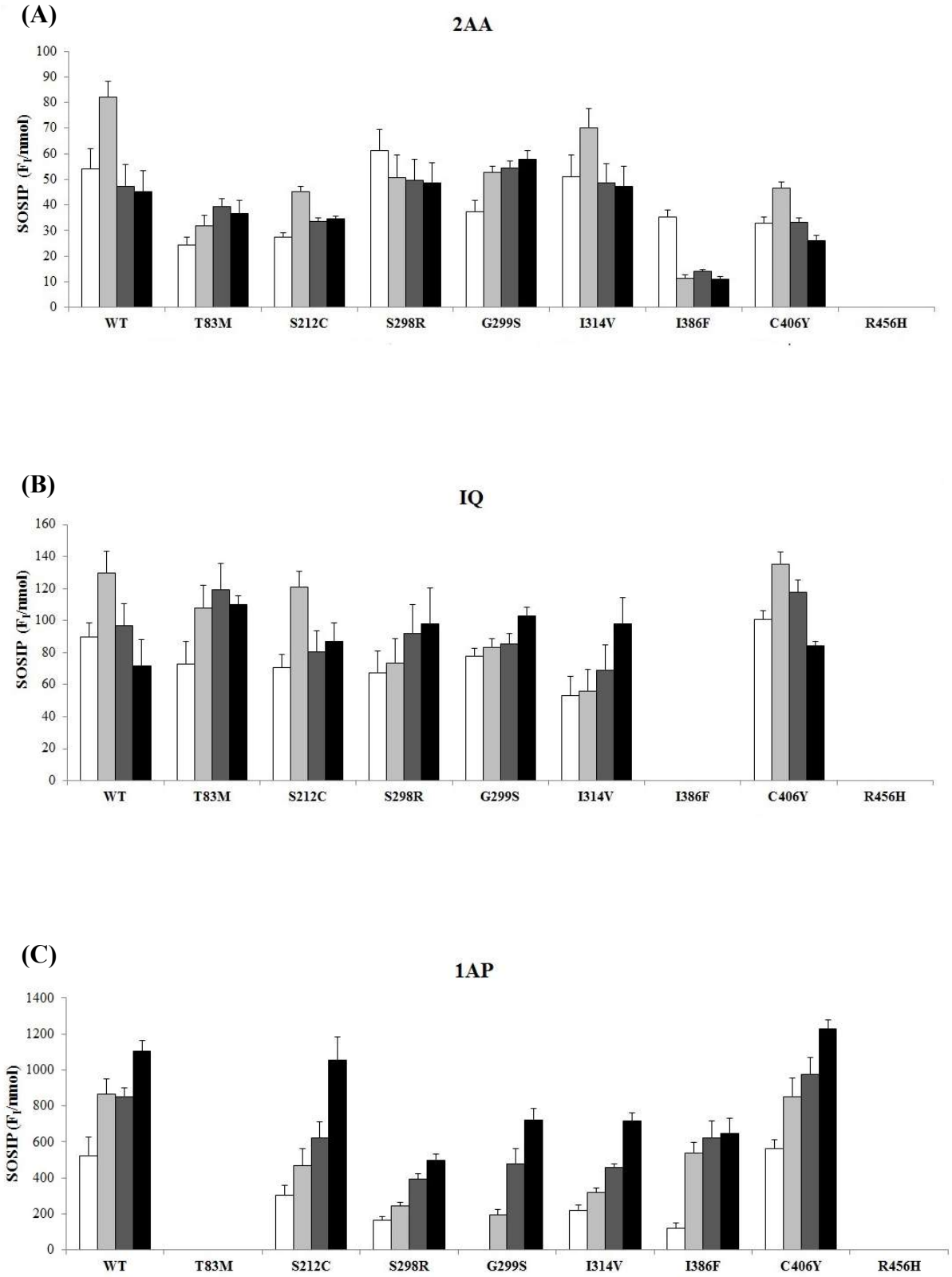

Figure 4. Histogram representing SOSIP values obtained with the TA1535 derived HT system, containing the different CYP1A2 variants and WT for 2AA (A), IQ (B) and 1AP (C) (column shading represents incubation times: white: 90 min; light gray: 120 min; dark gray: 150 min; black: 180 min).

5.3.3. Multivariate analysis of data

As in previous studies, the experimental data set (Supplemental Tables 1AC) was examined using several methods of multivariate analysis [29, 30]. (As variant R456H demonstrated no bioactivation activity, it was not included in the analysis.) Using the Pearson correlation with average linkage, a "clustering by similarity"-dendogram was obtained, based on the normalized data set (Fig. 5). Three classes of similarity were identified: one encompasses the T83M variant, the second one includes variants G299S and I386F, and the third CYP1A2 WT and the remaining variants. Variant T83M clearly has the least similar bioactivation activity when compared with the CYP1A2 WT, while variants G299S and I386F are slightly, but significantly, different from the CYP1A2 WT.

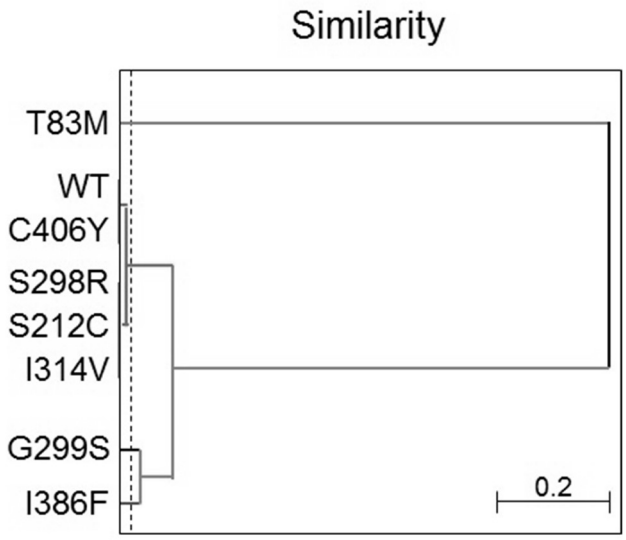

Figure 5. Clustering performed by similarity using the Pearson correlation with average linkage, for seven different CYP1A2 variants and CYP1A2 WT.

Principal component analysis (PCA) [38] is a multivariate analysis procedure which transforms a high number of possibly correlated variables into a reduced number of variables denominated by principal components. PCA was used to project the data set from a 12-dimensional space (SOSIP values at four different incubation times for the

three compounds) into a 2-dimensional space, for ease of visualization (Fig. 6). The PCA analysis retained 96% of the initial variance [principal component 1 (89%) plus component 2 (7%)]. Therefore, this analysis accounts for >95% of the variation in the data set. The PCA results clearly demonstrated variant T83M to be the most different variant when compared to CYP1A2 WT. The PCA analysis itself indicates that the variation stems primarily from the difference between variant T83M and the WT-like isoforms (WT, S212C, S298R, I314V, and C406Y), while variants G299S and I386F are only slightly different from CYP1A2 WT, as is demonstrated in Fig. 6.

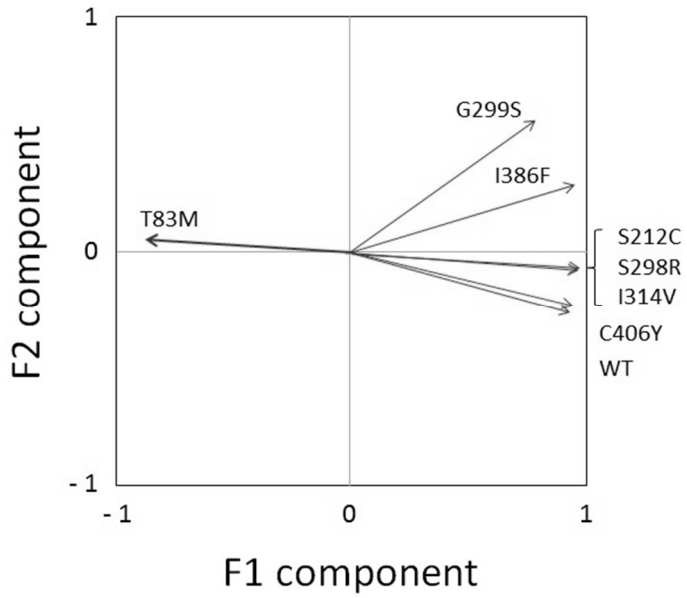

Figure 6. Gabriel plot of the PCA analysis for seven different CYP1A2 variants and WT. Normalized data-set for the first two principal components (96% of the initial variance retained).

Multidimensional scaling (MDS) [39] is a nonlinear multivariate analysis procedure which permits the visualization of the level of similarity between individual parameters in a data set. In the MDS configuration plot (Fig. 7), CYP1A2 variants were compared to their SOSIP values for the three pre-mutagens obtained at the four different incubation times. This plot shows the similarity/dissimilarity of the CYP1A2 variants in their bioactivation capacity of the three compounds. The correlation matrix was deduced

from the data set and a MDS was derived from this correlation matrix. The objects represent the variants scattered throughout the diagram, depending on their behaviour toward the three tested chemicals when taken globally. Two enzymes will be found close to each other in this MDS plot if and only if their behaviours toward the considered chemicals are similar. The Kruskal's stress is a diagnostic index that measures the closeness of the distance mapping in the MDS plot compared to the real distances in the multidimensional original space. The stress derived from the MDS plot (Fig. 7) is 0.032, indicating this analysis to be highly significant. When observing the MDS plot (Fig. 7), it is evident that the T83M variant is by far the least similar variant compared to the CYP1A2 WT and the separation of G299S and I386F from the rest of the variants is less marked in this analysis.

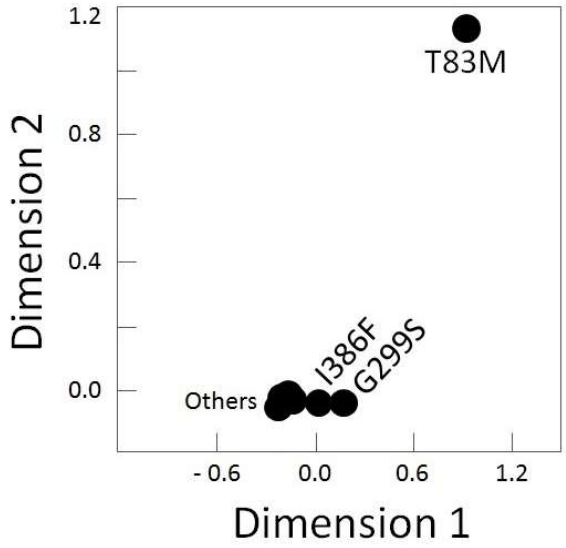

Figure 7. MDS configuration plot of seven different CYP1A2 variants and CYP1A2 WT.

5.4. DISCUSSION

Recently, we reported on the development of bacterial prototype systems for real-time detection of DNA reactive metabolites [31]. The systems are based on *S. typhimurium* or *E. coli* strains that express active recombinant human CYP1A2 and lend

themselves to adaptation to a HT assay format. The *S. typhimurium* TA1535-based system was selected for this current study versus the *E. coli* PD301-based system. Aromatic amines, such as 2AA, IQ, and 1AP, require CYP1A2-mediated oxidation and subsequent O-acetyl or O-sulfo conjugation to generate DNA-damaging metabolites [40]. The *S. typhimurium* tester strains of the Ames test, such as TA1535 [33], are known to express efficient bacterial O-acetyltransferases [41].

All CYP1A2 variants and the CYP1A2 WT expressed in the TA1535-derived system demonstrated similar CYP content, except for I386F and R456H. The I386F and R456H variants showed severely reduced or absent holoenzyme, respectively, in whole bacterial cells. Non-synonymous mutations in CYP polymorphic variants frequently lead to altered protein turnover. Although this cannot be determined accurately in bacterial cells, we found no changes in (apo)protein stability of the R456H and I386F variants, as described in our previous reports [29, 30]. Moreover, when expressed in the simian cell model COS-7, these variants demonstrated no significant alteration in protein stability [42]. The low or absent holoenzyme expression of R456H and I386F may be related to inefficient incorporation and/or anchoring of the prosthetic heme group, as indicated previously [29, 30]. The CPR- and CYP-expression levels in the new strains used in this study were comparable to those found in our previous studies [29, 31]. The CPR/CYP stoichiometry is recognized as an important factor for the catalytic properties of CYP enzymatic system [26]. The CPR/CYP ratio was constant in individual experiments, and daily values ranged (0.10-0.13) well within those observed for in human liver microsomes (0.08-0.5) [31]. These results indicate that co-expression of these CYP1A2 variants with CPR represents an adequate approximation of the in vivo situation, and that observed differences in CYP1A2 bioactivation activity could be solely ascribed to the structural deviations of the CYP1A2 variants [29].

Three CYP1A2 dependent promutagens, 2AA, IQ, and 1AP, were tested and their genotoxicity was detected using doses in the ng range. Variant R456H gave no detectable bioactivation activity for any of the compounds, as expected. Residue R456 is located adjacent to C457, the conserved cysteine (fifth ligand) involved in heme binding [43], as described in our former studies [29, 30]. In the case of variant I386F, which showed severely reduced levels of holoenzyme, no response to IQ was detected, but bioactivation of 2AA and 1AP was observed, albeit with decreased levels when compared to the CYP1A2 WT. The I386F substitution represents the replacement of an aliphatic side-chain with an aromatic side-chain in the substrate recognition site 5 (SRS-5) that could distort the heme-anchoring structure. We have previously shown the destabilization of the I386F holoprotein [29, 30].

The real-time measurement capacity of our HT-system allowed the consistent detection of differences in the time at which maximum SOSIP was reached among variants, for the three compounds (Figures 3A-C and Supplemental Table 1A-C). This indicates differences in the reaction velocity by which the genotoxic metabolites are formed by the different CYP1A2 variants. These differences represent additional information on the biotransformation/bioactivation efficacy of the CYP1A2 variants. As such, the data set used for multivariate analysis [38, 39] included the SOSIP values of all measured time points. The two applied statistical multivariate tools are of interest in several fields [44, 45] including the exploration of the protein structure space [46, 47].

All applied multivariate analyses had similar outcomes. Variant T83M was found to be the most divergent variant, when compared to CYP1A2 WT. The G299S and I386F variants also appeared significantly different from the CYP1A2 WT. All remaining variants (S212C, S298R, I314V and C406Y) could not be distinguished from the CYP1A2 WT. These results are in concordance with the recent report of Ito et al. [42]

describing the kinetic characterization of 20 allelic forms of CYP1A2, expressed in COS-7 cells, using de-ethylation of ethoxyresorufin and of phenacetin as probe reactions. For the six coinciding variants with our report, equivalent results were obtained, *i.e.*, variant R465H, no activity; variants T83M, G299S and I386F significantly different when compared with CYP1A2 wild type form; variants S212C and C406Y not significantly different from CYP1A2 WT. Of note, the Ito et al. study was based on a detailed kinetic characterization (V_{max}, K_M, CL_{int}) of the variants. Still, the determined efficacy parameter (CL_{int}: V_{max}/K_M) for the variants has a direct relation with the SOSIP values obtained for the three tested aromatic amines of this study, which reflects the efficacy of the variants to generate the DNA damaging metabolites. This is further confirmed, as activity evaluations coincide with those obtained in our previous study [29], investigating the same CYP1A2 variants but using eight diverse substrates, measuring 16 activity parameters (including V_{max} and K_M) for each variant. Although the initial objective of this current study was not to substitute characterization of polymorphic CYPs through determination of detailed kinetic parameters, the HT-assay and multivariate analysis seem to constitute a simplified method for the initial evaluation of CYP1A2 polymorphic variants.

Based on the human CYP1A2 structure published by Sansen and coworkers [32] (Supplemental Fig. 1), and the description of substrate entrance and product exit channels of CYPs by Cojocaru and coworkers [48] T83 is located in a substrate entrance/product exit channel. The T83M variant has demonstrated very different enzymatic activity from the WT in both our previous studies [29, 30]. It is interesting to note that, in the present work, the T83M variant did not bioactivate 1AP.

In the superposition and alignment of the human CYP1A2 crystal structure [32] with the structures of the CYP BM3 heme and FMN-binding domains [49], G299 is

located near the site of the CYPOR/cytochrome b_5 interaction. The secondary structural annotation of CYP1A2 (PDB #2HI4) also indicates that G299, along with S298, forms a beta hairpin structure. The G299S replacement could potentially disrupt the local fold through steric hindrance of the beta hairpin. The G299S variant was found to have very different enzymatic activity compared with CYP1A2 WT in our previous study of CYP1A2 variants [29]. Further investigation showed that in the presence of human cytochrome b_5 the G299S variant behaved like the CYP1A2 WT [30].

5.4.1. Conclusions

We expressed either CYP1A2 WT or one of eight variants with human CPR in the TA1535-derived prototype HT system and determined the effect of these variants on biotransformation activity for three well-known promutagens. Multivariate analysis indicated variant T83M as the most divergent from the CYP1A2 WT, while variants G299S and I386F are significantly different from the CYP1A2 WT, corroborating our previous studies. We demonstrated an application of our prototype HT system for the detection of genotoxic metabolites produced by CYP1A2 polymorphic variants, leading to the same conclusions reached in our previous studies [29, 30]. Moreover, these conclusions were reached with a substantially smaller data set. In this report we only used three CYP1A2 substrates versus eight CYP1A2 substrates (measuring 16 different activity parameters) in our previous studies [29, 30]. The current results indicate the effectiveness of the prototype HT system, not only for obtaining valuable information on the role of genetic CYP1A2 polymorphism on pre-mutagen bioactivation, but also for obtaining insights on the molecular mechanism underlying the functioning of this CYP isoform. This prototype HT system appears suitable for the study of structural

polymorphic variants of other human xenobiotic metabolizing CYPs, as these are readily expressed in *E. coli* [26-28] and *S. typhimurium* [50].

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5.5. SUPPLEMENTARY DATA

Table 1. SOSIP values obtained with the TA1535 derived HT system, containing the different CYP1A2 variants and WT for 2AA (A), IQ (B) and for 1AP (C)

(A)

	SOSIP[a]			
1A2 Variant	**90 min**	**120 min**	**150 min**	**180 min**
WT	54 ± 8	82 ± 6	47 ± 9	45 ± 8
T83M	24 ± 3	32 ± 4	39 ± 3	37 ± 5
S212C	27 ± 2	45 ± 2	34 ± 1	34 ± 1
S298R	61 ± 8	51 ± 9	50 ± 8	49 ± 8
G299S	37 ± 4	53 ± 2	54 ± 3	58 ± 3
I314V	51 ± 9	70 ± 8	48 ± 8	47 ± 8
I386F	35 ± 3	11 ± 1	14 ± 1	11 ± 1
C406Y	33 ± 3	47 ± 2	33 ± 2	26 ± 2
R456H	---	---	---	---

(B)

	SOSIP[a]			
1A2 Variant	**90 min**	**120 min**	**150 min**	**180 min**
WT	90 ± 9	130 ± 14	97 ± 14	72 ± 17
T83M	73 ± 14	108 ± 14	120 ± 16	110 ± 6
S212C	70 ± 9	121 ± 10	81 ± 13	87 ± 12
S298R	67 ± 14	73 ± 15	92 ± 18	98 ± 22
G299S	78 ± 5	83 ± 6	85 ± 7	103 ± 6
I314V	53 ± 12	56 ± 13	69 ± 16	98 ± 16
I386F	---	---	---	---
C406Y	101 ± 6	135 ± 8	118 ± 8	84 ± 3
R456H	---	---	---	---

(C)

	SOSIP[a]			
1A2 Variant	**90 min**	**120 min**	**150 min**	**180 min**
WT	521 ± 103	864 ± 87	850 ± 48	1106 ± 59
T83M	---	---	---	---
S212C	301 ± 57	466 ± 94	620 ± 94	1055 ± 126
S298R	164 ± 19	245 ± 19	392 ± 29	498 ± 35
G299S	0	195 ± 29	477 ± 84	722 ± 65
I314V	220 ± 27	320 ± 22	456 ± 22	718 ± 45
I386F	117 ± 33	536 ± 58	622 ± 93	648 ± 83
C406Y	563 ± 49	852 ± 101	974 ± 94	1229 ± 52
R456H	---	---	---	---

[a] Values are per nanomole (2AA and IQ) or per micromole (1AP) test compound and represent the mean ± SD; N ≥ 3. **---:** no response detected; values shaded in grey represent maximum SOSIP values.

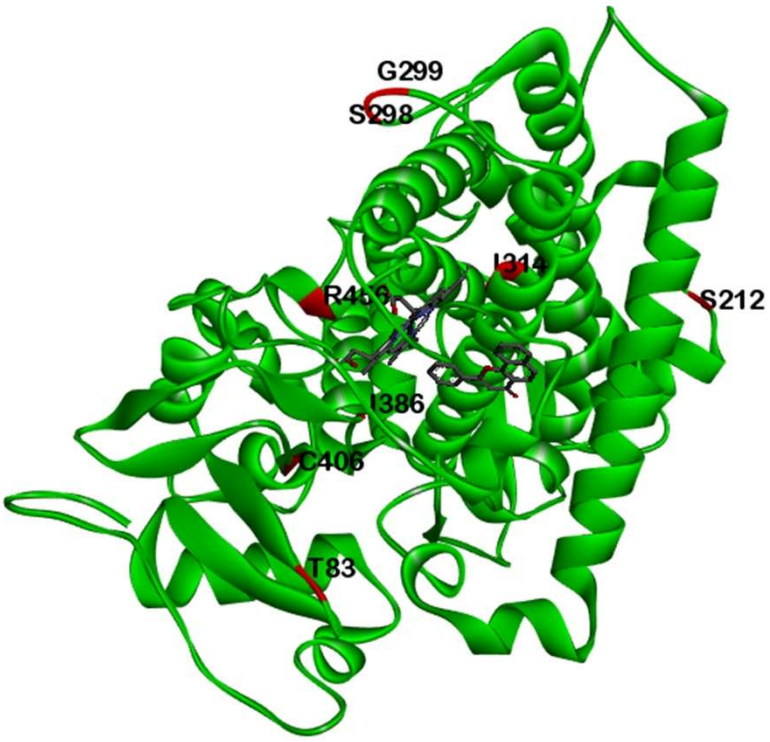

Figure 1. Localization of altered amino acids of the selected CYP1A2 polymorphic variants using the human CYP1A2 crystal structure published by Sansen *et al.* [1] (figure was generated using PDB file # 2H14, which contains the substrate α-naphtaflavone (αNF); heme and αNF in "ball-and -stick" representation).

5.5.1. References

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PART IV

CONCLUSIONS

CHAPTER 6

Summary, conclusions and perspectives

SUMMARY

Humans are continuously exposed to a variety of xenobiotics, such as dietary, pharmaceuticals, cosmetics, occupational or environmental chemicals. Most genotoxic carcinogens are chemically inert, requiring metabolic bioactivation by biotransformation enzymes to form chemically reactive metabolites (CRMs), which may covalently bind to DNA, RNA and proteins. A diversity of metabolic pathways have been recognized in the bioactivation of a variety of classes of carcinogens, with a key role of cytochrome P540 (CYP) enzymes [1]. In this context, the CYP1A family is of special interest, in bioactivating specific classes of chemical carcinogens and the development of cancer [2]. In fact, a single member of this family, namely CYP1A2, is responsible for the bioactivation of nearly 17% of known human pro-carcinogens [3], such as aromatic and heterocyclic amines [4]. Beside, CYP1A2 is involved in approximately 15% of the metabolism of clinical drugs [5].

Ever since the introduction of the revolutionary *S. typhimurium* mutagenicity assay, the "Ames test" over four decades ago, continuous efforts have been made on the engineering, improvement and application of additional bacterial-based genotoxicity assays [6]. However, the majority of the bacterial cell systems used for genotoxicity assessment lack xenobiotic biotransformation since they do not contain appropriate metabolic competences [7]. Moreover, at moment most of these assays are time consuming and/or demand many manipulation steps, thus not satisfying the current necessity of efficient high through-put screening (HTS). A possible approach to overcome these limitation is the engineering of new HTS-suitable bacterial systems with the stable introduction of expression vectors (cDNAs), encoding human biotransformation enzymes.

The *primary aim* of the research described in this thesis was to develop new bacterial genotoxicity test systems, being: i) competent in terms of human biotransformation and ii) suitable for HTS applications. The newly developed bacterial test systems should be suitable for genotoxicity and mechanistic studies, and for co-expression of different biotransformation enzymes and related polymorphic variants. The *secondary aim of the research* was to investigate the relevance of different CYP alleles of non-synonymous polymorphic forms. For these purposes, the characterization of their activity towards a diverse group of substrates should be studied, including the role of the accessory redox partner cytochrome b_5 (b_5). Human CYP1A2 and several CYP1A2 polymorphic variants were chosen as model enzymes for proof of concept.

Part I. Introduction

In **Part** I of this thesis, a general introduction to the work presented in this thesis is provided. At the end of **Chapter 1** the aims, in brief also described above, and an outline of this thesis are presented,

In **Chapter 1**, the pathways of xenobiotic biotransformation and the role of CRMs in genotoxicity and adverse drug reactions (ADRs) are discussed. An overview of the biotransformation enzymes involved in the bioactivation/bioinactivation of foreign compounds and the association between CRMs and toxicity in general, and genotoxicity in particular, is provided. Inter-individual differences in drug metabolism resulting from genetic polymorphisms of drug metabolizing enzymes, and their implications in the occurrence of ADRs and susceptibility to cancer is highlighted. The importance of the CYP superfamily, including the electron donor accessory enzymes b_5 and NADPH cytochrome P450 oxidoreductase (CYPOR), is explained with a special focus on the CYP1A2 isoform. Chemical carcinogenesis and mutagenesis, and the role of

bioactivation of xenobiotics is introduced. In this context, HTS-suitable *in vitro* assays for genotoxicity detection are discussed as well, with emphasis on bacterial systems.

In the early days, all HTS-suitable (yeast and bacterial) assays described at that time, applied external bioactivation systems (in majority rodent liver extracts) for the detecting of pro-mutagens. The primary aim of this PhD research was the development of bacterial genotoxicity test systems for HTS applications, competent in human biotransformation. Human CYP1A2, one of the major biotransformation enzymes involved in the metabolism of chemical carcinogens (*vide supra*) was selected as model for proof of concept.

Part II. Characterization of Human Cytochrome P450 1A2 Polymorphic Variants

Part II of this thesis deals with a comprehensive characterization of WT plus 8 non-synonymous polymorphic forms of human CYP1A2, namely T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H. These 8 naturally occurring variants were selected from a total of 15 (variants known in 2006, currently 24) non-synonymous polymorphisms (*http://www.cypalleles.ki.se/cyp1a2.htm*). This selection was based on the crystal structure published by Sansen *et al*. [8] of human CYP1A2 WT. Selection criteria for CYP1A2 variants concerned the localization of their structural deviations in the proximity of the active center, heme-moiety anchoring, substrate/product entrance/exit channel or the CYPOR interaction interface. The selection was performed with the assistance of MSc Eva Stjernschantz† and Dr. Chris Oostenbrink of the Division of Molecular Toxicology at the Vrije Universiteit Amsterdam.

At the start of this research, most of the work published about CYP1A2 polymorphisms was related to the characterization of gene polymorphisms in the non-

coding region. Few reports described non-synonymous variants, concerning the identification of polymorphisms in the population and their frequencies, but not the effects on protein level and activity. When activity was investigated, it was performed with a very limited number of substrates. In **Chapter 2**, a thorough characterization of the WT plus 8 non-synonymous polymorphic forms (variants indicated above) of human CYP1A2 is described for their metabolizing capacities. Each of the nine CYP1A2 variants was co-expressed with human CYPOR in *E. coli* BTC strains [9], using a bi-plasmid co-expression system. The CYP1A2 contents of variants were similar to WT, except for variants I386F and R456H. These two variants showed severely reduced or absent holoenzyme in whole cells, respectively. Neither of the two could be detected as holoenzymes in membrane (microsomal) fractions, however, immunoblot analysis indicated that CYP1A2 (apo-)-protein expression levels were similar among all variants. The CYPOR/CYP ratios for the different variants were found to be in the range of those observed in human liver microsomes, except for I386F and R456H, due to lack of CYP1A2 holoenzyme (*vide supra*). Activities of the CYP1A2 variants were determined, using 8 different substrates, using a large part of the known chemical space of CYP1A2's. These concerned 3 fluorogenic probes (MR, ER and CEC), 2 drugs (phenacetine and clozapine) and 3 pro-mutagens (NNK, IQ and 2AA). The resulting data set, consisting of 16 different activity parameters for each variant, was extensively analyzed using multivariate analysis methods. Variants R456H and I386F were not included in this analysis. These results were subsequently interpreted using the human CYP1A2 crystal structure. It was concluded that among all analyzed human CYP1A2 variants, variant G299S is the most deviated enzyme in comparison to WT, whereas variant T83M is only slightly but significantly different from the WT. Furthermore, bioactivation of NNK (mutagenicity) was shown to be the most discriminative activity among the CYP1A2

variants. In addition, R456 could be identified as a critical residue for proper heme anchoring and stabilization.

The investigation of the effect of the b_5 protein on the activities of WT plus the same 8 non-synonymous polymorphic forms of human CYP1A2 is described in **Chapter 3**. In this chapter, a similar approach, as reported in **Chapter 2** was applied, i.e. co-expressing separately the CYP1A2 variants with human CYPOR and human b_5, using the bi-plasmid system in the *E. coli* BTC strains [9, 10]. CYPOR contents and b_5 (holo- and apo-protein) expression levels were determined and were comparable among the strains. CYP1A2 variant contents, were comparable amongst variants (except for I386F and R456H) and similar to the ones described in **Chapter 2**, indicating that b_5 co-expression had minor effects on CYP expression. The CYP1A2 variants were screened using the same methods and substrates as described in **Chapter 2** determining the same 16 activity parameters. The results obtained were merged with the ones reported in **Chapter 2**. This combined data set was then analyzed through multivariate analysis and the structural knowledge derived from the human CYP1A2 crystal structure. Again, variants I386F and R456H were excluded from this analysis. Nevertheless, in the presence of b_5 variant I386F showed activity with all substrates, except with clozapine and IQ. Multivariate analysis indicated that among all human CYP1A2 variants analyzed, variants T83M and C406Y are the most divergent enzymes in catalytic properties in comparison with WT in b_5's presence. Collectively, data indicate that b_5 likely exerts a compensatory effect on the perturbed functional capacities of each of the polymorphic variants, leading to a more WT-like behavior. This is especially the case for the I386F variant, for which b_5 presence was essential for activity. Furthermore, the data seems to implicate CYP1A2 residue G299 in the interaction with CYPOR and/or b_5. It is concluded that b_5 can affect CYP1A2 polymorphic variants to behave more like the WT variant, seemingly through extensive

allosteric effects. These effects probably influence the active center (I386F), close to the CYPOR/CYP1A2 interface, but also more distal zones such as the substrate entrance/product exit channel (T83M and C406Y).

Part III. Development of Bacterial HTS-Systems for Detection of Genotoxic Electrophilic Reactive Metabolites

Part III of this thesis describes the engineering of bacterial systems that efficiently detect DNA-damaging electrophilic reactive metabolites generated by human CYP1A2 biotransformation.

In **Chapter 4**, the development of genotoxicity test systems, metabolically competent in human CYP1A2 and suitable for HTS purposes is presented. The principle of the detection method was based on a reporter fusion with the promotor sequence of *sulA*, one of the highest inducible bacterial genes upon DNA damage [11]. A specific GFP variant was selected as reporter, namely GFPmut3.1 [12]. This GFP was demonstrated to have one of the shortest maturation times and a high quantum yield, thus minimizing assay duration and enabling effective real-time measurements. An additional strain containing a constitutive promoter to correct for possible cytotoxic effects of test compounds was included, using the same GFP reporter. The reporter systems were initially used in four different bacterial backgrounds. These concerned TA1535 and TA100 [13], two *S. typhimurium* strains of the Ames test, and FP401[14] and PD301[9], two *E. coli* strains developed in our laboratory in Lisbon. After optimization of assay conditions, all reporter strains were tested with three direct-acting mutagens (4NQO, MNNG and CHP), to determine the sensitivity in genotoxicity detection of each of the 4 bacterial strains used. Results were compared with the ones of the SOS-chromotest [15]. Subsequently, the two most effective bacterial systems, namely the ones based on strains

TA1535 and PD301, were adapted to co-express human CYP1A2 and CYPOR, using the bi-plasmid system described previously, along with the GFP reporters. All strains demonstrated equivalent CYP and CYPOR levels and CYPOR/CYP ratios were within the range observed in human liver microsomes. The two systems were tested with three pro-mutagens, namely, 2AA, IQ and 1AP, well-known to form DNA-reactive metabolites through CYP1A2-mediated metabolism [16]. In **Chapter 4,** it is concluded that the two human CYP1A2 competent prototype systems, have the appropriate characteristics for HTS-detection of DNA-damaging reactive metabolites.

The application of the newly developed bacterial genotoxicity assay (described in Chapter 4) is reported in **Chapter 5**. This concerns the study of WT plus the same 8 polymorphic forms of human CYP1A2, studied in **Chapters 2** and **3**, using the HTS *S. typhimurium* TA1535 based system. CYP and CYPOR levels were comparable among all strains and CYPOR/CYP ratios within the range observed in human liver microsomes, except for the strains containing variants I386F and R456H, with expression issues as described in **Chapter 2** and **3.** Still, the bioactivation of two pro-mutagens by the I386F variant could be detected. The CYP1A2 dependent genotoxicity of the pro-mutagens 2AA, IQ and 1AP could be detected and results were analyzed through multivariate analysis. Variant T83M demonstrated again to be the most divergent isoform among all variants, while variants G299S and I386F were slightly but significantly different from WT. These results were comparable with those obtained in in **Chapter 2**, in which for each variant 8 different substrates (namely NNK, IQ, 2AA MR, ER, CEC, phenacetine and clozapine) were used and 16 different activity parameters were measured. This particular result indicated the effectiveness of the newly developed bacterial HTS system for genotoxicity, not only for investigation of the effect of CYP1A2 polymorphisms on

pro-mutagens bioactivation, but also to obtain insights on the molecular mechanism of CYP1A2 functioning.

FINAL CONSIDERATIONS, CONCLUSIONS AND PERSPECTIVES

Some Final Considerations

It is important to mention that the CYPOR/CYP ratios of all strains investigated in this thesis were always comparable with those found in human liver microsomes. This characteristic was considered important for *in vivo* significance. Moreover, the equivalence in CYP1A2 biotransformation capability of each variant, independent of the different *E. coli* and *S. typhimurium* strains used, permitted the conclusion that differences in detection of pro-mutagen genotoxicity is due to the bacterial background and not to differences in CYP biotransformation capacities (for **Chapter 4**); Conversely, when using the same bacterial background the differences in CYP1A2 biotransformation observed between the polymorphic CYP forms can be ascribed to differences between the variants (for **Chapters 2**, **3** and **5**).

In **Part III** of this thesis, the engineering of several novel *E. coli* and *S. typhimurium* tester strains, suitable for *in vitro* HTS-testing for xenobiotic genotoxicity, as well as for the study of 9 variants of human CYP1A2 is described. Two different types of plasmid-based GFP reporters were constructed, for the detection of genotoxicity and cytotoxicity, respectively. The usefulness of a cytotoxicity reporter strain (C-strains) was most apparent when testing CHP, a highly cytotoxic compound. The inclusion of the C-strains in HTS-testing is important to be highlighted, as is the very short time in data collection. The effectiveness of the HTS genotoxicity assay was successfully demonstrated as was the adaptation to a HTS-assay format, including microplate format,

requirement for small amount of test chemical, short turnaround time for data output, and real-time measurements.

All together, results of this thesis allow several important conclusions on specific aspects of the molecular functioning of the CYP1A2 enzyme complex, and the role of the accessory redox partner b_5 in their activity. In **Part II**, *E. coli* BTC [9] strains, developed in our lab in Lisbon, were chosen for mechanistic studies, because they were easily obtained through standard genetic manipulation of PD301, the mother strain of BTC. In **Part III,** when applying WT plus the 8 CYP1A2 variants in the novel genotoxicity bacterial assay, *S. typhimurium* strains were preferred.

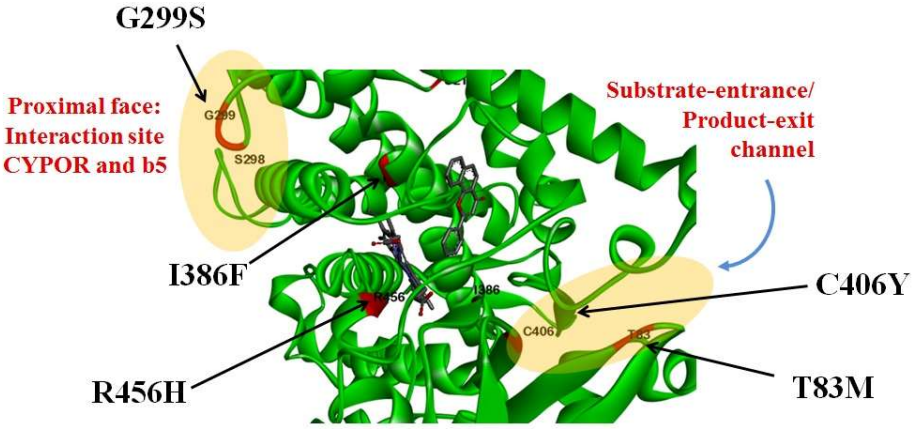

Figure 1. Localization of altered amino acids of the selected CYP1A2 polymorphic variants using the crystal structure of human CYP1A2, published by Sansen *et al* [8] (PDB file ID 2HI4). In pale yellow the substrate-entrance/product-exit and CYPOR/b_5 interaction zones are highlighted. Highlighted are also the most altered variants used in this thesis, namely: **T83M** and **C406Y**, which residues are suggested to be part of the architecture of the exit/entrance substrate/product channel(s). Substitutions of these residues may change substrate sampling and substrate orientation/positioning, leading to ineffective and/or alternative metabolism; **G299S**, located at the CYPOR/b_5 interaction zone, is forming a β-hairpin along with S298 (secondary structural annotation based on PBD file 2HI4); **I386F**, located in the SRS5, results in the replacement of an aliphatic side chain to an aromatic side chain, which may distort the heme anchoring architecture; **R456H**, residue R456 is adjacent to C457, the conserved cysteine (fifth ligand) involved in heme binding [17].

Variants I386F and R456H were demonstrated to be two particular forms with perturbed holoprotein stability. This observation was consistent in all studies in this thesis. The R456H substitution showed to be the most detrimental, since activity nor holoprotein levels could be detected. For variant I386F, when using whole bacterial cells, holoprotein and some activities were detected, while in membrane (microsomal)

preparations this was only the case when co-expressed with b_5. In fact, b_5 seems to improve protein stability and thus activity of this variant, seemingly through allosteric interactions.

Variant G299S is of special interest. In the absence of b_5, variant G299S was always the most divergent in catalytic properties, when compared to WT. However, in the presence of b_5, its activity profile was very much like the WT. The results obtained clearly implicate G299 in CYP1A2's interaction with the overlapping CYPOR/b_5 binding site. Interestingly, the G299S substitution seems to be more disruptive to the local structure than the adjacent S298R substitution, which showed WT like activities throughout the different studies.

Variants T83M and C406Y demonstrated the most surprising results, i.e. substantially altered catalytic properties of both these polymorphic forms when compared to the WT variant. Moreover, the altered activity profiles of both T83M and C406Y in the presence of b_5 (**Chapter 3**) seem the emphasize their divergence, as all other variants behaved in a more WT-like manner. The only exception, was in **Chapter 2**, where variant C406Y was considered as a WT-like enzyme. However, the same work also showed a lack of NNK bioactivation, while in phenacetine metabolism, beside the dealkylation product extra metabolites were observed. Interestingly, Zhou *et al.* (2004) postulated a possibly altered metabolic profile for variant C406Y [18].

Conclusions and Perspectives

Biotransformation and related inter-individual differences, play a major role in chemical genotoxicity, carcinogenicity and individual susceptibilities. CYP metabolism is usually the main clearance pathway for drugs and other xenobiotics. [19, 20]. Metabolic activation, i.e. bioactivation of xenobiotics sometimes results in the formation of CRMs,

known to be a crucial determinant in multiple toxicities (such as ADRs) in general, and in genotoxicity in particular [21, 22]. Although the generation of CRMs is not an absolute indicator that exposure to a compound will result in toxicity, the ultimate action of CRMs is also dependent on the efficiency of various metabolic detoxification routes and the presence of toxicological targets at the site of CRM formation [23]. Absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) guided lead-optimization is often based on a strategy of avoiding compounds having a potential to produce CRMs [24].

The initial tests of chemicals for their potential to induce carcinogenesis, usually depends on genotoxicity or mutagenicity testing. This is currently particularly relevant due to the implementation of the EU chemical policy REACH, with a requirement for thousands of toxicity profiles of non-characterized or poorly characterized chemicals [25]. The screening of thousands of compounds using whole animal models is neither economically viable nor ethically acceptable, thus reinforcing the need for new, highly effective HTS (geno)toxicity screening methods [26]. In this context, bacterial *in vitro* cell-models are of interest due to their simplicity in manipulation, short term in data collection and the fact that they are in line with the societal concern on laboratory animal use. However, the inadequate representation and/or absence of human biotransformation in such *in vitro* assays is still a major handicap of *in vitro* genotoxicity test systems when relevant CRMs occur and for *in vitro* to *in vivo* extrapolations [27].

The need for improved *in vitro* genotoxicity HTS-testing has been addressed by several laboratories with the development of new test systems. However, some can only be considered as “semi” HTS, while appropriate human biotransformation incorporation is still the general handicap in all cases. The work described in this thesis, addressed these particular issues.

We designed and engineered the novel SOS-CYPtest system. This system is using several *E. coli* and *S. typhimurium* tester strains, which are metabolically competent in human CYP biotransformation and are appropriate for *in vitro* HTS genotoxicity testing of chemicals, which was *the primary aim* of this study. The SOS-CYPtest system strains were engineered to include a GFP reporter, and to express an active human CYP (CYP1A2) and several of its polymorphic forms. In the first phase, the ability to detect DNA-damaging agents was demonstrated. Subsequently, the genotoxicity of CYP1A2-dependent pro-mutagens was successfully detected. Although only applied for a restricted number of compounds, the newly developed SOS-CYPtest system seems to have the appropriate characteristics for the detection of DNA damaging CRMs in a HTS manner. HTS characteristics include a short assay time (max 120-180 min), the need of only small amounts of test compound (less than a few micrograms), the adaptability to a microplate format and real-time measurement. Besides the sensitive and efficient screening of a large number of chemicals, obtaining relevant data on the role of particular human CYP isoforms in the genotoxicity of chemicals, as another example of its applicability, the SOS-CYPtest system was also tested to study genetically polymorphic variants of human CYPs.. It was demonstrated not only to generate successfully genotoxicity data, relevant for risk assessment and individual susceptibility in the exposure to chemical carcinogens, but also to yield new insights in the molecular mechanisms of function of human CYP1A2 variants. In Figure 2, a graphical illustration is given to visualize the different test systems developed, the expressed proteins involved, the probe substrates used, the pro-mutagens used as well as the flow schemes used to the ultimate applications in terms of risk assessment and mechanistic information.

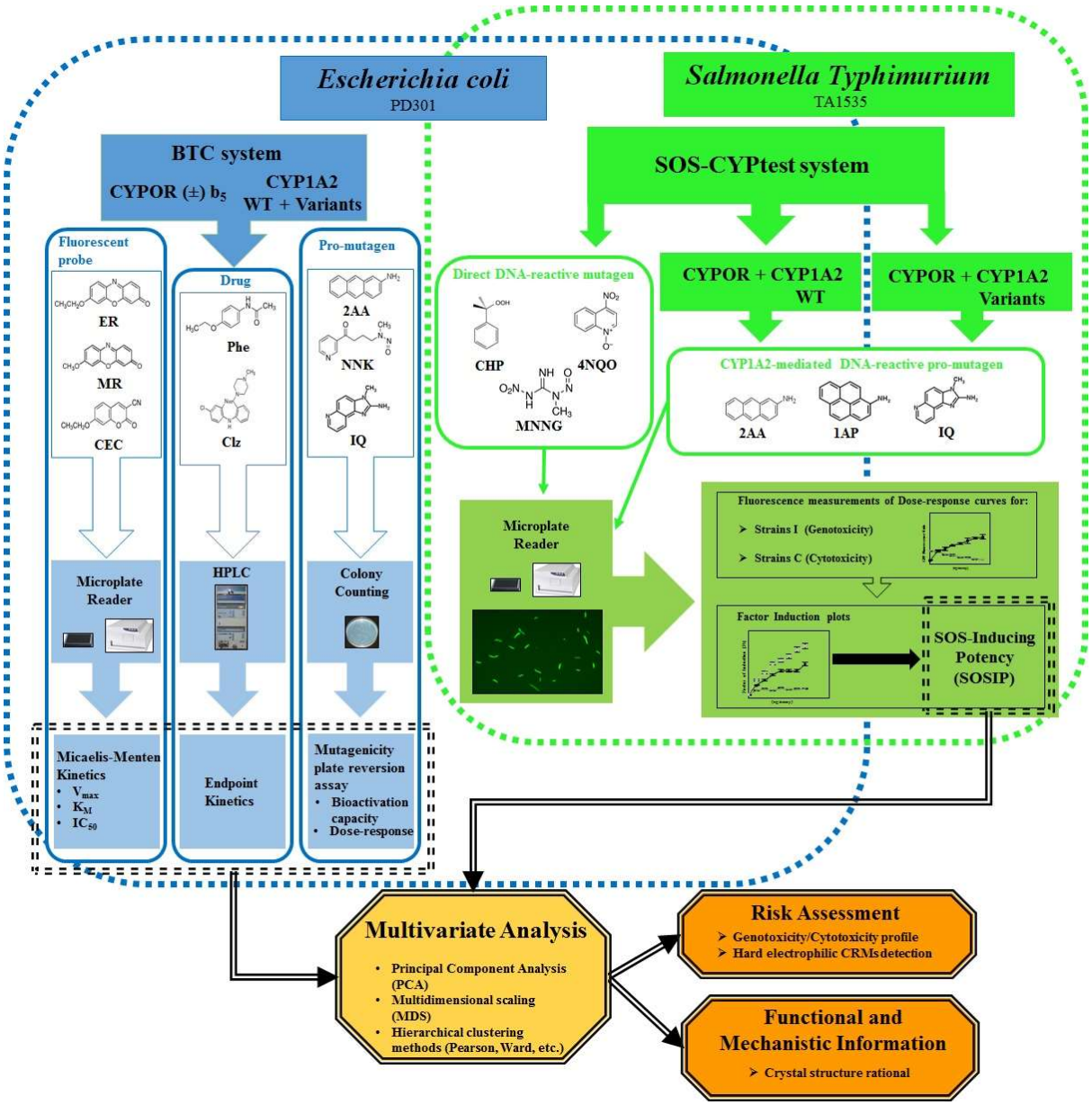

Figure 2. Graphical summary of the research described in this thesis. For the development of the novel SOS-CYPtest system, two bacterial backgrounds were used, namely *E. coli* PD301 strains [14] and *S. typhimurium* TA1535 strains [13]. Both backgrounds were used for the detection of 3 direct DNA-reactive mutagens, namely: CHP, cumene hydroperoxide; 4NQO, 4-nitroquinoline-1-oxide; MNNG, N-methyl-N´-nitro-N-nitrosoguanidine. In the SOS-CYPtest system, CYPOR and WT CYP1A2 were co-expressed in both bacterial backgrounds, while the CYP1A2 variants were only co-expressed in *S. typhimurium* TA1535 strains. Three CYP1A2-mediated DNA-reactive pro-mutagens were tested, namely: 1AP, 1-aminopyrene; 2AA, 2-aminoanthracene; and IQ, 2-amino-3-methylimidazo(4,5-*f*)quinoline. For mechanistic studies of CYP1A2 variants, the *E. coli* BTC system [9] was used. Several techniques were applied, namely Michaelis-Menten kinetics and inhibition assays, endpoint HPLC kinetics and mutagenicity plate reversion assays. In total 9 different compounds representing different classes of CYP1A2 substrates were used, namely: 3 fluorogenic probes (ER, ethoxyresorufin; MR, methoxyresorufin; and CEC, 3-cyano-7-ethoxycoumarin), 2 therapeutic drugs (phenacetine and clozapine) and 4 pro-mutagens [1AP; 2AA; IQ; and NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone]. To scrutinize appropriately all results, multivariate analysis methods were applied.

The novel bacterial system, the SOS-CYPtest system, was designed and engineered to offer a HTS test system that would accurately detect which compounds can produce DNA-reactive CRMs in humans. Systems for detection of DNA damaging CRMs do not *per se* require the complexity of the eukaryotic or mammalian nucleus. The use of such complex systems might even diminish or impair effective detection of some CRMs. Moreover, the short cell cycle of bacteria allows a fast turnaround in data output (2-3 hrs), as demonstrated in this thesis. Comparable HTS assays using yeast or mammalian cells, require more than 16 hrs. Our systems are effective in detecting hard genotoxic electrophilic CRMs generated by biotransformation enzymes, such as the CYPs. However, detoxification of CRMs by other metabolic enzymes, such as e.g. GSTs, is not present in the current systems and consequently the test systems may overestimate CRM exposure. Thus, the use of the standard Ames plate assays combined with human liver S9, representing bioactivation and bioinactivation, might be more adequate for risk assessment, however, not for identifying CYP-isoenzyme selective metabolic pathways and related variability. Therefore, the identification and capability in generating genotoxic metabolites by CYP-isoenzymes of chemicals remains of great relevance. This is also the case when detoxification pathways would become saturated (e.g. at high levels of exposure or in the case of drug-drug interactions) or in cases where the detoxification capacity is limited (e.g. due to genetic polymorphisms of genes encoding detoxifying enzymes).

The *secondary aim* of this thesis was to investigate specific CYP alleles, focusing on non-synonymous polymorphic forms, and the role of the accessory enzyme b_5. CYP1A2 was chosen as a model enzyme. Due to the huge amount of data produced, and to be able to scrutinize appropriately all results, multivariate analysis methods were applied and appeared to be a powerful and very useful tool regarding data analysis.

Collectively, it is concluded that 5 of the 8 different CYP1A2 variants analyzed were of relevance, namely variants T83M, G299S, I386F, C406Y and R456H. Consistently, R456H was an inactive enzyme. T83M and C406Y are the most divergent enzymes comparing to WT CYP1A2, while variant I386F is a very unstable enzyme in which b_5 plays a key role in its stability/activity. The results obtained clearly implicate G299 in CYP1A2 to interact with CYPOR/b_5. Collectively, b_5 seems to compensate for structural deviations of the CYP1A2 variants, most likely through extensive allosteric effects. Not only the proximal zones of the active center and the b_5/CYPOR/CYP1A2 interface are affected, but also more distal zones such as the substrate entrance/product exit channel.

In conclusion, this thesis describes the design and the engineering of a new SOS-CYPtest system, a bacterial assay for the real-time detection of *in situ* generated DNA reactive metabolites, and competent for human CYPs, i.e. the most relevant enzyme in xenobiotic human biotransformation. The SOS-CYPtest systems were developed to address a specific limitation of current *in vitro* HTS-genotoxicity tests, namely the lack of human biotransformation. The novel SOS-CYPtest systems are also suitable for HTS screening formats. Thus, the test strains described in this thesis, constitute a new generation of human biotransformation competent tester bacteria effective for testing genotoxic compounds for risk assessment, but also for mechanistic studies, e.g. on the molecular mechanisms underlying the functioning of CYP enzymes and their polymorphic forms. In this thesis CYP1A2 has been used to provide a proof of concept.

In terms of future perspectives, the currently developed SOS-CYPtest systems, are prototypes that can be optimized and further specialized. Subsequent efforts should preferably be dedicated to the validation of assay(s) including genotoxins with different

mechanisms, but also including non-genotoxic compounds. This in order to challenge the test systems as much as possible, and also to proceed with optimizations for commercial purposes. Other human CYP isoforms could also be applied or even other biotransformation enzymes such as NQOs, SULTs and GSTs, all leading to bioactivation to and/or protection against CRMs. Several of these enzymes have already been expressed in bacteria [28-31]. The expression of other CYPs (and their polymorphic forms) especially the most relevant for pro-carcinogens bioactivation, namely CYPs 1A1, 1B1, 2A6, 3A4 is of special interest [3].

The integrated statistical multivariate methods are of great use for analysis of this type of data, and thus for improving the quality of interpretations and extrapolations both for risk assessment and/or functional/mechanistic studies. Extended design and engineering of the genotoxicity assays *per se,* e.g. by refining the reporter and cellular systems, may also be considered. In this context, the integration in the bacterial genome of two reporters (notably for cytotoxicity and genotoxicity) can be envisioned, ideally even considering placement of both reporters in the same target cell. Similarly, gene-reporters could be applied to monitor other effects of xenobiotics, such as oxidative stress. Our test systems are representing only CYP1A2-mediated metabolism, however, one might also envision simultaneous co-expression of different CYP isoforms and/or other biotransformation enzymes (phase I, II and III) trying to get closer to the *in vivo* situation. The use of different promotors for each enzyme might even be considered, e.g. to vary levels of expression for mechanistic purposes or to better represent inter-individual differences. Alternatively, one could use mixtures of different reporter bacteria, each containing different CYP isoforms and/or other biotransformation enzymes (phase I, II and III). Such reporter bacteria could be mixed in relative quantities mimicking specific metabolism in tissues (e.g. liver, lung, cardiac tissues, etc.). Such approaches might also

represent CYP- and/or other enzymes, (such as GSTs) mediating detoxification routes of parent compounds and/or metabolites. The use of other bacterial backgrounds and/or the use of other indicative reporter bacteria together with a bioreactor could also be explored. The current bacterial genotoxicity strains could be the point of departure for these further developments.

In xenobiotic risk assessment, the potency to induce carcinogenesis is usually provided by genotoxicity or mutagenicity testing. The major handicap of current *in vitro* HTS-systems to detect relevant DNA-reactive metabolites is mostly due to inadequate representation and/or failure of human biotransformation. The studies in this thesis demonstrate possibilities to overcome this issue. Mechanistic information regarding human biotransformation enzymes is also generated. Application and further engineering of the current *in vitro* HTS-methods to study genotoxicity can contribute to improved selection of safer drug candidates in pharmaceutical R&D, but also for the identification of pollutants and/or hazardous environmental and occupational agents.

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APPENDICES

SUMMARY

Humans are continuously exposed to a variety of xenobiotics, such as, pharmaceuticals, cosmetics, dietary, occupational or environmental chemicals. Most genotoxic carcinogens are chemically inert, requiring metabolic bioactivation by biotransformation enzymes to form chemically reactive metabolites (CRMs), which may subsequently covalently bind to DNA, RNA and proteins. A diversity of metabolic pathways has been recognized in the bioactivation of carcinogens, with a key role of Cytochrome P540 (CYP) enzymes. In this context, the CYP1A family is of special interest in the bioactivation of specific classes of chemical carcinogens and in the development of cancer.

Ever since the introduction of the revolutionary *S. typhimurium* mutagenicity assay, the "Ames test" over four decades ago, continuous efforts have been made on the improvement and application of additional bacterial-based genotoxicity assays. However, the majority of the available bacterial cell systems used for genotoxicity assessment still lack xenobiotic biotransformation, since they do not contain appropriate metabolic competences.

The *primary aim of the research* described in this thesis was to develop new bacterial genotoxicity test systems, being: i) competent in terms of human CYP-mediated biotransformation and ii) suitable for HTS applications. The bacterial test systems should be suitable for genotoxicity and mechanistic studies, and for co-expression of different CYP enzymes and related polymorphic variants. The *secondary aim of the research* was to investigate the relevance of different CYP alleles of non-synonymous polymorphic forms. For these purposes, their activity towards a diverse group of genotoxic and non-genotoxic substrates should be studied, including the role of the accessory redox partner

Cytochrome b_5 (b_5). Human CYP1A2 and several polymorphic CYP1A2 variants were chosen as model enzymes for proof of concept.

Part I. Introduction

In **Chapter 1**, the pathways of xenobiotic biotransformation and the role of CRMs in genotoxicity and adverse drug reactions (ADRs) are discussed. An overview of the biotransformation enzymes involved in the bioactivation/bioinactivation of foreign compounds and the association between CRMs and toxicity in general, and genotoxicity in particular, is presented. Inter-individual differences in drug metabolism resulting from genetic polymorphisms of drug metabolizing enzymes, and their implications for ADRs and susceptibility to cancer is highlighted. The importance of the CYP superfamily, including the electron donor accessory enzymes b_5 and NADPH cytochrome P450 oxidoreductase (CYPOR), is explained with a special focus on CYP1A2. Chemical carcinogenesis and mutagenesis, and the role of bioactivation of xenobiotics are introduced as well. In this context, HTS-suitability of *in vitro* assays for genotoxicity detection, with emphasis on bacterial systems, is discussed as well.

Part II. Characterization of Human Cytochrome P450 1A2 Polymorphic Variants

Part II of this thesis deals with a comprehensive characterization of WT plus 8 non-synonymous polymorphic variants of human CYP1A2, namely T83M, S212C, S298R, G299S, I314V, I386F, C406Y and R456H; and the role of the accessory redox partner cytochrome b_5.

In **Chapter 2**, a thorough characterization of the WT CYP1A2 plus 8 non-synonymous polymorphic variants (indicated above) is described for their metabolic capacities. Each of the eight CYP1A2 variants plus WT was co-expressed with human CYPOR in *E. coli* strains, using a bi-plasmid co-expression system. All variants showed

similar levels of apoprotein and holoprotein expression, except for I386F and R456H, which showed only apoprotein. However, both were functionally inactive. The CYPOR/CYP ratios for the different variants were found to be in the range of those observed in human liver microsomes, except for I386F and R456H, due to lack of CYP1A2 holoenzyme (*vide supra*). Activities of the CYP1A2 variants were determined, using 8 different substrates. The resulting data set, consisting of 16 different activity parameters for each variant, was extensively analyzed using multivariate analysis methods. These results were subsequently interpreted using the human CYP1A2 crystal structure. It was concluded that among all analyzed human CYP1A2 variants, G299S is the most deviating enzyme in comparison to WT, whereas variant T83M is only slightly but significantly different from the WT. Furthermore, bioactivation of NNK (mutagenicity) was shown to be the most discriminative activity among the CYP1A2 variants. In addition, R456 could be identified as a critical residue for heme anchoring and stabilization.

In **Chapter 3**, the effect of the b_5 protein on the activities of WT CYP1A2 plus the same 8 non-synonymous polymorphic forms is described. A similar approach, as reported in **Chapter 2** was applied. CYPOR contents and b_5 (holo- and apo-protein) expression levels were determined and comparable among the strains. CYP1A2 variant levels, were comparable amongst variants (except for I386F and R456H) and similar to the ones described in **Chapter 2**, indicating that b_5 co-expression had minor effects on CYP expression, in this model. The CYP1A2 variants were screened using the same methods and substrates as described in **Chapter 2**. The results obtained were merged with the ones reported in **Chapter 2**. The combined data set was then analyzed through multivariate analysis and the structural knowledge derived from the human CYP1A2 crystal structure. This indicated that among all human CYP1A2 variants analyzed,

variants T83M and C406Y are the most divergent enzymes in catalytic properties when compared to WT in b_5's presence. Collectively, data indicate that b_5 likely exerts a compensatory effect on the perturbed functional capacities of the polymorphic variants, leading to a more WT-like behavior. Furthermore, the data seems to implicate CYP1A2 residue G299 is in interaction with CYPOR and/or b_5.

Part III. Development of Bacterial HTS-Systems for Detection of Genotoxic Electrophilic Reactive Metabolites

Part III of this thesis describes the engineering of bacterial systems to efficiently detect DNA-damaging CRMs generated by human CYP1A2.

In **Chapter 4**, the development of genotoxicity test systems, metabolically competent in human CYP1A2 and suitable for HTS purposes is presented. The principle of detection was based on a reporter fusion with the promotor sequence of *sulA* (one of the highest inducible bacterial genes upon DNA damage) with GFPmut3.1, a selected GFP reporter. An additional strain containing a constitutive promoter to correct for possible cytotoxic effects of test compounds was included, using the same GFP reporter. The reporter systems were initially used in four different bacterial backgrounds, namely TA1535 and TA100: two *S. typhimurium* strains of the Ames test, and FP401 and PD301, two *E. coli* strains developed in our laboratory in Lisbon. After optimization of assay conditions, all 4 reporter strains were tested with three direct-acting mutagens (4NQO, MNNG and CHP), to determine the sensitivity in genotoxicity detection. Subsequently, the two most effective bacterial systems, namely the ones based on strains TA1535 and PD301, were adapted to co-express human CYP1A2 and CYPOR, using the bi-plasmid system described previously, along with the GFP reporters. All strains demonstrated equivalent CYP and CYPOR levels and CYPOR/CYP ratios were within the range observed in human liver microsomes. The two systems were tested with three pro-

mutagens, namely, 2AA, IQ and 1AP, known to form DNA-reactive metabolites through CYP1A2-mediated metabolism.

The application of the newly developed bacterial genotoxicity assay (described in Chapter 4) for HTS application is reported in **Chapter 5**. This concerns the study of WT CYP1A2 plus the same 8 polymorphic forms, studied in **Chapters 2** and **3**, and using the *S. typhimurium* TA1535 based HTS system. CYP and CYPOR levels were comparable among all strains and CYPOR/CYP ratios were within the range observed in human liver microsomes, except for the strains containing variants I386F and R456H, with the expression issues described in **Chapter 2** and **3.** Still, the bioactivation of two pro-mutagens by the I386F variant could be detected. The CYP1A2-dependent genotoxicity of the pro-mutagens 2AA, IQ and 1AP could be detected and the results were analyzed through multivariate analysis. Variant T83M demonstrated again to be the most divergent isoform from WT among all variants, while variants G299S and I386F were slightly but significantly different from WT. These results were comparable with those obtained in in **Chapter 2**, in which for each CYP1A2 variant 8 different substrates (namely NNK, IQ, 2AA MR, ER, CEC, phenacetine and clozapine) were used and 16 different activity parameters were measured. This particular outcome indicated the effectiveness of the newly developed bacterial HTS system for genotoxicity testing, not only for investigation of the effect of CYP1A2 polymorphisms on pro-mutagens bioactivation, but also for providing insights in the molecular mechanism of CYP1A2 functioning.

Conclusions and perspectives

We designed and engineered the novel SOS-CYPtest system. This system is using several *E. coli* and *S. typhimurium* tester strains, which are competent in human CYP metabolism and are appropriate for *in vitro* HTS genotoxicity testing of chemicals, which was *the primary aim* of this study. Although only applied for a restricted number of

compounds, the newly developed SOS-CYPtest system seems to have the appropriate characteristics for the detection of DNA damaging CRMs in a HTS manner. HTS characteristics include a short assay time (max 120-180 min), the need of only small amounts of test compound (less than a few micrograms), the adaptability to a microplate reader format and real-time measurement.

The *secondary aim* of this thesis was to investigate specific CYP alleles, focusing on non-synonymous polymorphic forms, and the role of the accessory enzyme b_5. CYP1A2 was chosen as a model enzyme. Due to the huge amount of data produced, and to be able to scrutinize appropriately all results, multivariate analysis methods were applied and appeared to be a powerful and very useful tool regarding data analysis. All-together, it is concluded that 5 of the 8 different CYP1A2 variants analyzed, namely variants T83M, G299S, I386F, C406Y and R456H, were of relevance in terms of altered metabolism. Consistently, R456H was inactive. T83M and C406Y were the most different enzymes when compared to WT CYP1A2, while variant I386F is a very unstable enzyme in which b_5 plays a key role in its stability/activity. The results obtained clearly implicated G299 in CYP1A2 to interact with CYPOR/b_5. Collectively, b_5 seems to compensate for structural deviations of the CYP1A2 variants, most likely through extensive allosteric effects. Not only the proximal zones of the active center (I386F) and the b_5/CYPOR/CYP1A2 interface (G299S) are affected, but also more distal zones such as the substrate entrance/product exit channel (T83M and C406Y).

In xenobiotic risk assessment, the potency to induce carcinogenesis is usually provided by genotoxicity or mutagenicity testing. The major handicap of current *in vitro* HTS-systems is mostly due to inadequate representation and/or failure of human biotransformation. The studies in this thesis demonstrate possibilities to overcome this issue. Mechanistic information was also generated. Application and further engineering

of the current *in vitro* HTS-methods to study genotoxicity can contribute to improved selection of safer drug candidates in pharmaceutical R&D, but also for the identification of pollutants and/or hazardous environmental and occupational agents.

SUMÁRIO

Os seres humanos são continuamente expostos a uma variedade de xenobióticos tais como, produtos farmacêuticos, cosméticos, compostos presentes nos alimentos, agentes químicos ocupacionais ou ambientais. A maioria dos carcinogéneos genotóxicos são quimicamente inertes, requerendo bioativação metabólica pelos enzimas de biotransformação para formar metabolitos quimicamente reativos (CRMs), que posteriormente se podem ligar covalentemente a DNA, RNA e proteínas. Na bioactivação de carcinogéneos, foram reconhecidas diversas vias metabólicas, tendo os enzimas do Citocromo P540 (CYP) um papel fundamental. Neste contexto, a família CYP1A tem especial interesse na bioactivação de classes específicas de agentes químicos cancerígenos e no desenvolvimento de cancro.

Desde a introdução há mais de quatro décadas do "teste de Ames", um ensaio revolucionário de mutagenicidade em *S. typhimurium*, têm sido realizados esforços contínuos para a melhoria e aplicação de novos ensaios de genotoxicidade baseados em bactérias. No entanto, a maioria dos sistemas celulares bacterianos atualmente disponíveis para a avaliação da genotoxicidade ainda não possuem sistemas de biotransformação de xenobióticos, uma vez que não contêm as competências metabólicas apropriadas.

O objetivo principal da investigação descrita nesta tese foi o desenvolvimento de novos sistemas bacterianos para testar genotoxicidade, sendo estes: i) competentes em termos de biotransformação mediada por CYP humano e ii) adequados para aplicações em high-throughput screening (HTS). Os sistemas de teste bacterianos devem ser adequados para estudos de genotoxicidade, estudos mecanísticos, e para co-expressão de diferentes enzimas CYP e das suas variantes polimórficas. O objetivo secundário foi investigar a relevância de diferentes alelos polimórficos não-sinónimos de CYP. Para tal, a sua actividade foi investigada com recurso a um grupo diversificado de substratos

genotóxicos e não genotóxicos, incluindo o papel do Citocromo b_5 (b_5), um parceiro redox acessório. Para prova de conceito, CYP1A2 humano e várias variantes de CYP1A2 polimórficas foram escolhidos como enzimas modelo.

Parte I. Introdução

No Capítulo 1, são discutidas vias de biotransformação xenobiótica e o papel dos CRMs na genotoxicidade e nas reações adversas a medicamentos (ADRs). É apresentada uma visão geral dos enzimas de biotransformação envolvidos na bioativação/bioinativação de xenobióticos, e a associação entre CRMs e toxicidade em geral e genotoxicidade em particular. São destacadas as diferenças inter-individuais no metabolismo de fármacos resultantes de polimorfismos genéticos nos enzimas de metabolização, e suas implicações para ADRs e susceptibilidade ao cancro. A importância da superfamília de CYP, incluindo os enzimas acessórios dadores de electrões, b_5 e NADPH citocromo P450 oxidoredutase (CYPOR), é explicada, com foco especial em CYP1A2. É também discutido o papel da bioactivação de xenobioticos na carcinogénese e mutagénese. Neste contexto, a adequabilidade de ensaios de genotoxicidade para uso em contexto de HTS é discutida, com enfase em sistemas bacterianos.

Parte II. Caracterização de Variantes Polimórficas do Citocromo P450 1A2 Humano

A **Parte II** desta tese aborda a caracterização aprofundada de CYP1A2 humano WT e de 8 variantes polimórficas não-sinónimas, nomeadamente: T83M, S212C, S298R, G299S, I314V, I386F, C406Y e R456H; bem como o papel do parceiro redox citocromo b_5.

No **Capítulo 2**, é descrita uma caracterização abrangente das capacidades metabólicas de CYP1A2 WT e das 8 variantes polimórficas não-sinónimas (indicadas acima). Cada uma das oito variantes de CYP1A2 mais WT foi co-expressa em conjunto com CYPOR humano em estirpes de *E. coli,* utilizando um sistema bi-plasmídico de co-expressão. Todas as variantes apresentaram níveis semelhantes de expressão de apoproteína e holoproteína, com excepção de I386F e R456H, onde se observou apenas a presença de apoproteína. Desta forma, ambas as variantes são funcionalmente inativas. Os rácios CYPOR/CYP encontrados para as diferentes variantes foram equivalentes aos observados em microssomas hepáticos humanos, exceto para I386F e R456H, devido à falta de holoenzima CYP1A2 (*vide supra*). As actividades das variantes de CYP1A2 foram determinadas, utilizando 8 substratos diferentes. O conjunto de resultados obtido, composto por 16 parâmetros de actividade diferentes para cada variante, foi extensamente analisado com recurso a métodos de análise multivariada. Estes resultados foram posteriormente interpretados usando a estrutura cristalina de CYP1A2 humano. Concluiu-se que, entre todas as variantes de CYP1A2 humana analisadas, a variante G299S é o enzima mais divergente em relação ao WT, enquanto a variante T83M é apenas ligeiramente, mas significativamente diferente da variante WT. Adicionalmente, a bioactivação do composto NNK (mutagenicidade) demonstrou ser a actividade mais discriminativa entre as variantes de CYP1A2 analisadas. Além disso, o R456 pôde ser identificado como um resíduo crítico para a ancoragem e estabilização do heme.

No **Capítulo 3**, é descrito o efeito do b_5 nas actividades de CYP1A2 WT e das mesmas 8 formas polimórficas não-sinónimas. Foi aplicada uma abordagem semelhante à relatada no **Capítulo 2**. Os teores de CYPOR e os níveis de expressão de b_5 (holo- e apo-proteína) foram determinados, sendo comparáveis entre estirpes. Os níveis de expressão de CYP1A2 encontrados foram comparáveis entre as variantes (excepto para

I386F e R456H) e semelhantes aos descritos no **Capítulo 2**, indicando que a co-expressão de b_5 não tem grande impacto na expressão de CYP. As variantes CYP1A2 foram testadas com os mesmos métodos e substratos descritos no **Capítulo 2**. Os resultados obtidos foram fundidos com os relatados no **Capítulo 2**. Este conjunto de dados combinados foi então avaliado através de análise multivariada, tendo em conta o conhecimento estrutural derivado da estrutura cristalina de CYP1A2 humano. Concluiu-se que, na presença de b_5, de entre todas as variantes humanas de CYP1A2 analisadas, as variantes T83M e C406Y são os enzimas mais desviantes em propriedades catalíticas quando comparadas à WT. Colectivamente, os dados indicam que provavelmente b_5 exerce um efeito compensatório sobre as capacidades funcionais alteradas das variantes polimórficas, levando a um comportamento mais próximo da variante WT. Adicionalmente, os dados parecem implicar o resíduo G299 de CYP1A2 na sua interacção com CYPOR e/ou b_5.

Parte III. Desenvolvimento de Sistemas HTS Bacterianos para Detecção de Metabolitos Reactivos Electrofílicos Genotóxicos

A **Parte III** desta tese descreve a engenharia de sistemas bacterianos para detecção eficiente de CRMs DNA-reactivos gerados pelo CYP1A2 humano.

No **Capítulo 4**, é apresentado o desenvolvimento de sistemas de teste de genotoxicidade, metabolicamente competentes em CYP1A2 humano e adequados para HTS. O princípio da detecção baseou-se na fusão de um repórter com a sequência do promotor de *sulA* (um dos genes bacterianos mais induzíveis após dano de DNA), com GFPmut3.1, um repórter GFP. Foi incluída uma estirpe adicional contendo um promotor constitutivo para corrigir possíveis efeitos citotóxicos dos compostos de teste, utilizando o mesmo repórter GFP. Os sistemas repórteres foram inicialmente utilizados em quatro estirpes bacterianas diferentes, nomeadamente TA1535 e TA100: duas estirpes de *S.*

typhimurium do teste de Ames, e FP401 e PD301, duas estirpes de *E. coli* desenvolvidas no nosso laboratório em Lisboa. Após a optimização das condições de ensaio, as quatro estirpes foram testadas com três mutagénios de acção directa (4NQO, MNNG e CHP), para determinar a sensibilidade do ensaio na detecção de genotoxicidade. Posteriormente, os dois sistemas bacterianos mais eficazes, nomeadamente os baseados nas estirpes TA1535 e PD301, foram adaptados para co-expressar CYP1A2 humano e CYPOR, utilizando o sistema bi-plasmídico descrito anteriormente, conjuntamente com os repórteres GFP. Todas as estirpes demonstraram níveis equivalentes de CYP e CYPOR, estando os rácios CYPOR/CYP dentro do intervalo observado em microssomas hepáticos humanos. Os dois sistemas foram testados com três pró-mutagénios, nomeadamente, 2AA, IQ e 1AP, conhecidos por formar metabolitos DNA-reactivos através de metabolismo mediado por CYP1A2.

No **Capítulo 5** é relatada a aplicação HTS do ensaio de genotoxicidade bacteriana recém-desenvolvido (descrito no **Capítulo 4**). Foram utilizados CYP1A2 WT mais as 8 formas polimórficas (estudadas nos **Capítulos 2** e **3**), aplicando o sistema de HTS baseado em *S. typhimurium* TA1535. Os níveis de CYP e CYPOR foram comparáveis entre todas as estirpes e os rácios CYPOR/CYP estavam dentro do intervalo observado nos microssomas hepáticos humanos, com excepção das estirpes contendo as variantes I386F e R456H, que apresentaram os mesmos problemas de expressão descritos nos **Capítulos 2** e **3**. Ainda assim, pôde ser detectada a bioactivação de dois pro-mutagénios pela variante I386F. A genotoxicidade dos pró-mutagénios 2AA, IQ e 1AP dependente de CYP1A2 pôde ser detectada, tendo os resultados obtidos sido analisados através de análise multivariada. A variante T83M demonstrou novamente ser a isoforma mais divergente da WT entre todas as variantes, enquanto as variantes G299S e I386F foram ligeira, mas significativamente, diferentes da WT. Estes resultados foram comparáveis

aos obtidos no **Capítulo 2**, no qual, para cada variante CYP1A2, foram utilizados 8 substratos diferentes (NNK, IQ, 2AA MR, ER, CEC, fenacetina e clozapina) medindo 16 parâmetros de actividade. Este resultado demonstrou a eficácia do sistema HTS bacteriano recém-desenvolvido para testes de genotoxicidade, não apenas para investigar o efeito de polimorfismos de CYP1A2 na bioactivação pró-mutagénica, mas também para fornecer informações sobre o mecanismo molecular do funcionamento de CYP1A2.

Conclusões e Perspectivas

Com a realização desta tese desenhámos e desenvolvemos o novo sistema SOS-CYPtest. Este sistema utiliza várias estirpes de teste de *E. coli* e *S. typhimurium*, tornadas metabolicamente competentes em CYP humano e apropriadas para testar *in vitro* a genotoxicidade de químicos em HTS, sendo este o principal objectivo deste estudo. Embora tenha sido testado com apenas um número restrito de compostos, o novo sistema SOS-CYPtest parece ter as características apropriadas para a detecção de CRMs DNA-reactivos, no formato HTS. Estas características HTS incluem um tempo de execução curto (120-180 min max.), a necessidade de pequenas quantidades de composto de teste (menos de alguns microgramas), a capacidade de adaptação ao formato de um leitor de microplacas e a medição em tempo real.

O objectivo secundário desta tese foi investigar alelos específicos de CYP, com foco em formas polimórficas não-sinónimas e no papel do enzima acessório b_5. CYP1A2 foi escolhido como um enzima modelo. Devido à enorme quantidade de dados gerados, e de forma a poder analisar todos os resultados adequadamente, foram aplicados métodos de análise multivariada, que demonstraram ser uma ferramenta poderosa e muito útil na análise de dados. No conjunto, conclui-se que 5 das 8 variantes diferentes de CYP1A2 analisadas, nomeadamente as variantes T83M, G299S, I386F, C406Y e R456H, foram as

mais relevantes em termos de alteração no metabolismo. A variante R456H foi consistentemente inactiva. T83M e C406Y foram os enzimas mais divergentes em comparação com WT, enquanto a variante I386F é um enzima muito instável na qual b_5 desempenha um papel fundamental na estabilidade/actividade. Os resultados obtidos implicaram claramente o resíduo G299 de CYP1A2 na interacção com CYPOR/b_5. Colectivamente, b_5 parece compensar os desvios estruturais das variantes de CYP1A2, muito provavelmente através de efeitos alostéricos. Não apenas as zonas proximais do centro activo (I386F) e a interface b_5/CYPOR/CYP1A2 (G299S) são afectadas, mas também outras zonas distais, como o canal de entrada do substrato/saída do produto (T83M e C406Y).

Na avaliação de risco de xenobióticos, a capacidade para induzir a carcinogénese é geralmente averiguada com recuso a testes de genotoxicidade ou mutagenicidade. A principal desvantagem dos atuais sistemas HTS *in vitro* deve-se à representação inadequada e/ou falha da biotransformação humana. Os estudos nesta tese descrevem possibilidades para superar essa questão. Informações mecanísticas foram também geradas. A aplicação e o aperfeiçoamento do actual sistema *in vitro* para estudar genotoxicidade, pode contribuir para melhorar a investigação e desenvolvimento farmacêuticos na selecção de candidatos a medicamentos mais seguros, bem como para a identificação de poluentes e/ou agentes ambientais e ocupacionais perigosos.

CURRICULUM VITAE

Bernardo J.E. de Brito Palma was born on November 15th 1979 in Lisboa, Portugal. In 1997 he graduated from high-school at Escola Secundária D. Pedro V in Lisboa, Portugal. In the same year, he started studying Chemistry at the Faculty of Sciences of the Universidade de Lisboa. In 1999, he changed subject from Chemistry to Biochemistry within the same institution. From September 2002 to October 2003 he completed the curricular one year research internship in the Department of Genetics of the Faculty of Medical Sciences of the Universidade Nova de Lisboa, under the supervision of Prof. Dr. José Rueff and Dr. Michel Kranendonk. The research project was entitled "Development of new bacteria strains to study xenobiotic biotransformation: Co-expression of Human Cytchrome b_5, Cytchrome P450 and NADPH-Cytchrome P450 reductase, to use in mutagenicity assays". In October 2003, he received his *Licentiati Gradum* (5 years degree, MSc equivalent) in Biochemistry at the Faculty of Sciences, Universidade de Lisboa. From October 2003 to January 2004, he performed a post-graduate research period in the same project under the same supervision. From February 2004 to July 2004, he did a post-graduate research period in the project entitled "Site-Directed Mutagenesis of cytochrome P450 1A2", in the Division of Molecular Toxicology, Department of Pharmacochemistry, Vrije Universiteit Amsterdam under the supervision of Prof. Dr. Nico P.E. Vermeulen and Dr. Barbara van Vugt-Lussenburg. In 2006, using a grant from the *Fundação para a Ciência e Tecnologia*, Portugal (SFRH/BD/23038/2005), he initiated his PhD project entitled "Development of Human Cytochrome P450 Competent Genotoxicity Tester bacterial Systems for High Throughput Screening: Functional Characterization of Human Cytochrome P450 1A2 Polymorphic Variants" in a collaboration between the Faculty of Medical Sciences of the Universidade Nova de Lisboa and the Division of Molecular Toxicology at the Vrije

Universiteit Amsterdam, under the supervision of Prof. Dr. Nico P.E. Vermeulen and Prof. Dr. José Rueff, and co-supervision of Dr. Michel Kranendonk.

In 2011 he obtained the CAP certificate (Trainer Certificate), by the Institute of Employment and Professional Training from the Portuguese Ministry of Employment and Social Solidarity, Portugal.

From 2011 to 2013 he held a Lecturer position in the Instituto Superior de Ciências da Saúde Egas Moniz (ISCSEM), Almada, Portugal. He co-lectured "Forensic Toxicology" within the Forensic and Criminal Sciences BSc degree, at ISCSEM and part at the Department of Forensic Toxicology of the National Institute of Legal Medicine and Forensic Sciences, Portugal. He also co-lectured "Xenobiochemistry", within the Erasmus Mundus Forensic Science Master Degree (ISCSEM, Portugal, Universidad de Cordoba, Spain and University of Lincoln, UK).

Since 2013 he is a resident trainer at AdLaser, a company responsible for the module "Radiation Interaction with eyes and skin" within the "Advanced Laser Safety Training Course - Application to Industry".

Also since 2013, he is a researcher in the Centre for Toxicogenomics and Human Health (ToxOmics), NOVA Medical School, Universidade NOVA de Lisboa (former CIGMH, Faculty of Medical Sciences of the Universidade Nova de Lisboa).

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"Let the snows fall deep, the rain drive down, and the wind buffet my cloak. I care not, for I've a road worth walking!"

Drizzt Do´Urden (R.A. Salvatore)